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CANCER OF THE LUNG IN RELATION TO TOBACCO

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Further Data on the Arsenic Content of Cigarettes.

In an earlier paper (Daff and Kennaway, 1950) data were given for the arsenic content of 5 brands of cigarettes of British and American types, of 8 brands of Turkish type, and of two others (French, Rhodesian), and also for the amount of arsenic volatilized in smoking. A further series of estimations of arsenic in cigarettes from 8 countries (U.S.A., Canada, Norway, Switzerland, Austria, Italy, Bulgaria, Poland) is reported in Table I. In all, 27 brands have been examined.

All quantities of arsenic given in this paper are stated as As_2O_3 μg/g., which is numerically the same as parts per million.

The results given in this and the earlier paper show—

(1) A high arsenic content (24 to 106) in 10 non-Turkish brands smoked in England, U.S.A., Canada and Norway.

(2) A low arsenic content (0·0 to 4·3) in 8 Turkish brands as sold in England, in a Rhodesian brand, in a popular Austrian brand, and in cigarettes from France, Poland and Bulgaria.

(3) Intermediate amounts (3·1 to 12·0) in a less popular Austrian brand, and in cigarettes from Switzerland and Italy. One Canadian brand showed a range of 8·6 to 18·7.

Of course no claim is made that the analyses of these cigarettes, which we have happened to obtain through the kindness of various friends, can give any complete picture of the comparative habits of smokers in the different countries. But most of the brands are popular ones and the results show, on the whole, a gradation from the arsenic-rich American type in Western Europe to the arsenic-poor Turkish type in the East, with an intermediate mid-European zone, which accords with Hutson's (1937) account of tobacco culture in Europe quoted below (p. 3).

Cancer of the Lung in Relation to the Types of Tobacco Smoked in Various Countries.

Some recent investigations, and especially those of Wynder and Graham (1950), and of Doll and Bradford Hill (1950), show an association between the smoking of tobacco, and especially of cigarettes, and cancer of the lung. Since arsenic is under

TABLE I.—*Arsenic Content of Cigarettes. Brands other than English.*

Brand.	Number of cigarettes.	As_2O_3 μg. per g.
U.S.A.		
Brand C	2	46·4 ; 46·7.
,, P.M.	1	51·0.
Canada.		
Brand K	4	36·8 ; 58·2 ; 47·1 ; 55·1.
,, O	5	8·7 ; 18·4 ; 8·6 ; 18·7 ; 9·1.
,, W	6	41·1 ; 46·5 ; 57·9 ; 79·5 ; 67·5 ; 41·7.
,, B.C.	3	43·5 ; 57·6 ; 42·5.
Norway		
Brand B	3	71·7 ; 65·8 ; 74·6.
,, T	4	54·4 ; 52·4 ; 55·6 ; 49·7.
Switzerland.		
Brand P	4	6·4 ; 3·1 ; 3·9 ; 3·4.
Italy.		
Brand N	6	10·5 ; 9·8 ; 10·2 ; 13·7 ; 9·1 ; 8·3.
Austria.		
Brand D	1	12·0.
,, M	3	Trace ; 2·1 ; 0·9.
Poland.		
Brand Z	5	2·5 ; 2·1 ; 1·2 ; 2·7 ; 1·5.
Bulgaria.		
Brand A	4	0·7 ; 1·2 ; nil ; 0·3.
,, R	4	0·6 ; 0·6 ; nil ; 0·2.

some circumstances carcinogenic in man (Neubauer, 1947 ; Currie, 1947 ; Bradford Hill and Faning, 1948), the question arises, whether the arsenic in tobacco is concerned in this process. Instances of cancer of the lung in workers exposed to arsenic are given by the Chief Inspector of Factories (1939). In a case of arsenical poisoning " . . . due to sodium arsenite, which was fatal, the post-mortem examination revealed that in addition to pigmentation of the trunk and limbs, warty growths all over the body and perforation of the nasal septum, there was a primary cancer of the right lung with metastatic growths in neighbouring glands and in the liver." In a later Report (Chief Inspector of Factories, 1943) another case is recorded " . . . in a filling machine operator, aged 57, for 43 years in a factory manufacturing sheep dip containing sodium arsenite, the cause of death being due to carcinoma of the right lung. Three similar cases of

pulmonary carcinoma occurring in arsenical sheep-dip workers have been notified since 1939." Henry (1950) describes a case of cutaneous cancer " . . . in a furnaceman making sodium arsenite who eventually died of cancer of the lung . . ." Two non-industrial cases of bronchial cancer are recorded in persons taking arsenic medicinally over long periods (Montgomery and Waisman, 1941; Semon, 1945). This question might be decided if one could have equally reliable statistics for the incidence of cancer of the lung in countries where the predominant tobacco smoked is of the arsenic-rich, or of the arsenic-poor, type. We have been collecting data on this question by correspondence, but this is a slow and difficult process. In view of the great practical importance of the matter it seems better to publish the available data now in the hope of promoting first-hand inquiry in appropriate countries.

So far as we know, the only statistical studies of cancer of the lung in relation to cigarette smoking which have come from any countries other than Great Britain, the U.S.A. and Germany are those of Saglam (1944) from Turkey and of Dungal (1950) from Iceland.

The data which one would like to obtain from any given country are:

A. *From national sources.*—(1) Total consumption of tobacco. (2) Proportion of tobacco which is of American, or Turkish, type. (3) Proportion of tobacco smoked as cigarettes, cigars and pipe-tobacco. (4) Population by ages. (5) Number of deaths attributed to cancer of the lung. The two last items for men and women separately.

B. *From university clinics* (for men and women separately—preferably by ages).—(1) Number of autopsies. (2) Number of autopsies showing cancer. (3) Number of autopsies showing cancer of lung.

In the countries where A(5) is not available one must rely on B. The ratio of B(3) to B(1) has been suggested as the most reliable index for the present purpose (Heady and Kennaway, 1949). Figures for a number of separate years are, of course, very desirable, but those for the war period 1940 to 1945 generally show considerable disturbances (Fig. 2, 5) and must be avoided. We are indebted to the staffs of various Embassies in London for data on A(1), (2), (3) and (4), in addition to what can be found in such works of reference as the *Statesman's Year Book*. A great deal of information is given in a report, "The Consumption and Production of Tobacco in Europe," by J. B. Hutson (1937).

In such comparisons between different countries at least four possible carcinogenic factors must be considered, namely: (1) Smoking of tobacco. (2) Smoking of tobacco containing arsenic. (3) Inhalation of the dust of towns and of coal smoke. (4) Inhalation of products of the internal combustion engine.

Thus populations in eastern Europe which are subject to the first of these factors, and only slightly if at all to the second, will also be comparatively free from the third and fourth, while the industrial peoples of Britain and the U.S.A. will be most exposed to the second, third and fourth together, and the inhabitants of Iceland will be subject to the second only.

Tobacco Culture in Europe.

Hutson (1937) classifies the types of tobacco grown in Europe thus:

(1) Oriental: Turkey, Greece, Bulgaria, S. Yugoslavia, U.S.S.R.

(2) Semi-Oriental: S.E. Italy, S.W. Yugoslavia, Rumania, Hungary, S. Czechoslovakia, S. Poland.

(3) Dark air-cured : Hungary, Rumania, Czechoslovakia, Germany, N. Italy, France, Spain, Belgium, Sweden, Switzerland.

He divides the countries of Europe into 4 groups :

(1) Those in which a portion of the factory consumption is grown (Germany, Spain, Belgium, Poland, Czechoslovakia, Switzerland).

(2) Those in which production is approximately equal to consumption (Italy, Yugoslavia, Rumania).

(3) Those which produce a surplus (Greece, Turkey, Bulgaria, Hungary).

(4) Those in which little or no tobacco is grown, which are all those not named above.

The countries in the third group are of most interest, if one can assume that the tobacco smoked there is at any rate very largely of the arsenic-poor type, but unfortunately the conditions for the study of cancer of the lung in the whole population are not as yet very favourable in them, and this subject, like that of cancer of the liver, presents peculiar difficulties on account of the danger of confusion of primary and secondary growths.

Tobacco Consumption and Cancer of the Lung in Various Countries.
Turkey.

(1) Data on the consumption of tobacco in Turkey are given in Table II.

TABLE II.—*Consumption of Tobacco in Turkey.*

Year.	Tobacco consumption (million lb.)	Population.	Tobacco consumption (lb. per head.)
1925	15·8*	13,023,000	1·21
1930	21·9*	14,591,000	1·50
1935	25·0*	16,158,018	1·55
1937	28·2†	16,800,000	1·68
1942	37·2†	17,820,950	2·09
1947	40·7†	18,870,785	2·16
1949	39·7†	20,900,009‡	1·90

* Figures from Hutson (1937).
† Figures provided by the Turkish Embassy.
‡ *The Times*, October 30, 1950.

(2) Prof. Schwartz, of the University of Istanbul, writes that in Turkey tobacco is consumed almost wholly in the form of cigarettes ; that cigars, European pipes and the oriental water-pipe are almost unknown among the bulk of the people, and that there are many heavy smokers among women.

(3) The only publication from any Balkan country upon cancer of the lung in relation to smoking appears to be that of Prof. Saglam (1944) of the University of Istanbul, who gives the following details, which are quoted in full in view of the importance of his original paper, and the difficulty of access to it.

" The clinical statistics of pulmonary carcinoma in Turkey :

	Year.	Number of patients.	Pulmonary tumour.	Rate per thousand.
The Int. Clinic of Gulhane (Suleyman Numan)	1899–1908	4270	2	0·46 (0·47)*
The Int. Clinic of Gulhane (Tevfik Saglam)	1923–1926	2343	7	2·1 (3·0)*
The Sect. for Int. Diseases of Gureba (Tevfik Saglam)	1931–32	1459	9	7·1 (6·2)*
Haydarpaxa Numune Hast. (Tevfik Saglam)	1936–1939	4230	24	5·6 (5·7)*

* Recalculated from the figures given by Prof. Saglam in the two adjacent columns.

"The anatomo-pathologic statistics:

	Year.	Number of autopsies.	Pulmonary cancer.	Rate per thousand.
Gulhane (Deycke-Reinhard)	1899–1908	800	3	3·7
Gulhane (Lufti)	1923–1926	502	5	10
Haydarpasa Numune Hast.	1936–1939	635	14	22
The Anatomo-pathologic Institute of the University of Istanbul (Prof. Ph. Schwartz)	1935–1943	5126	79	15·4

"Taking the clinical statistics into consideration it is clear that within 30–40 years the increase in our clinics is 12 times and according to the anatomo-pathologic statistics it is 4·1 times as much."

"We have seen 11 cases of pulmonary carcinoma in the newly inaugurated 3. Ic Hastaliklari Klinigi in 23 months (among 2084 patients, 5·3 in a thousand)."

"All these statistics show that in our country, within the last 30 years, pulmonary carcinoma has increased in a great proportion."

Saglam rejects cigarette-smoking as a factor in the incidence of bronchial carcinoma. "Some authors believe that there exists a relationship between smoking tobacco and inhaling the smoke and pulmonary carcinoma. Animal experiments seem to support this point of view. Some authors are of the opinion that especially cigarettes play a great part in its production. We do not hold the same view. For a long time, at least since 50 years, almost only cigarettes are smoked in our country. In spite of this the increase in pulmonary Ca. in this country has gone on the same pace as in those countries where cigars and pipes are mostly smoked. The amount of tobacco consumed in our country has shown a constant increase, and from 9,370,000 kgr. in 1926 has risen to 16,680,000 kgr. in 1942. But this rise is not sufficient to explain the increase in pulmonary Ca."

But there is no exact basis for any such argument. We do not yet know what consumption of any tobacco produces any given incidence of bronchial carcinoma, nor do we know the time-relations of any such effect.

(4) We are indebted to Prof. Saglam, Prof. Schwartz and Dr. Yenermen for (*a*) full protocols of the 193 autopsies on cases of primary tumour of the lung carried out at the University of Istanbul during 1934–1950, and (*b*) figures for the total autopsies and autopsies on cases of cancer, by sexes, for the same period (Table III).

To make the material comparable with other series, we have omitted the

TABLE III.—*Cases of Cancer of the Lung Among Autopsies Carried Out at the University of Istanbul*, 1935–1950.

Period.	Number of autopsies.								
	All cases.			All cancer.			Cancer of lung.		
	Male.	Female.	Total.	Male.	Female.	Total.	Male.	Female.	Total.
1935–9	1833	947	2780	217	97	314	33	5	38
1940–4	2585	747	3332	302	70	372	48	7	55
1945–9	2612	1323	3935	359	129	488	73	10	83
1949–50	*767*	*488*	*1255*	*104*	*53*	*157*	*31*	*4*	*35*

Changes in incidence will be seen more clearly in Table IX, where cases of cancer of the lung are shown as percentages of all autopsies and of all cancers. Note that data for the year 1949 appear twice.

data for 1934 and have removed 3 cases, all in men, described as benign tumours, from the total of primary lung tumours (a chondroma in 1935, another in 1939, and a polyp in 1937). We have, however, retained four cases described histologically as not being carcinoma, namely, lymphogranuloma in a man, one case each in 1936 and 1937 ; sarcoma in a man, 1941 ; cylindroma in a woman, 1942.

Bulgaria and Greece.

Hutson (1937) states that the surplus produced in Bulgaria and Greece, with that from Turkey and Hungary, makes up the bulk of the world's supply of oriental cigarette tobacco. It is difficult to estimate the amount consumed locally as an unknown amount is grown for personal use and escapes tax. The official figures for tobacco consumption (Hutson, 1937) and for population (*Statesman's Year Book*, 1931) indicate an annual consumption of about 1·7 lb. per head in Greece and of 1·9 lb. per head in Bulgaria, in the period 1925 to 1930. The figures are similar to those obtained for Turkey in 1937 (1·68 lb. per head, Table II).

We have no data on the incidence of cancer of the lung in these countries.

Yugoslavia.

We do not know the amount of tobacco consumed in Yugoslavia, but Dr. Kosir, of Ljubljana, has provided us with information about the smoking habits of the population. He writes that the kinds of tobacco smoked in Yugoslavia are : " (*a*) type Makedonija (cigarettes) ; (*b*) type Hercegovina (cigarettes) ; and (*c*) type Vojcodina (pipe and cigars)."

" There are smoked principally cigarettes. These are of the oriental type, i.e., they have a natural ' bouquet ' which gives the tobacco type Makedonija." . . . Only in the north-western part of Yugoslavia there are smoked cigars and pipe. Use of tobacco for snuffing is quite insignificant. . . ." Before the last war the only import was some Dutch tobacco for cigars ; during the war some Bulgarian and after it some American tobacco (through U.N.R.R.A.) was imported, but all these amounts were very small.

We are also indebted to Dr. Kosir and to Prof. F. Hribar for a statement of the numbers of autopsies and of those on all cases of malignant disease and of cancer of the lung, by sex, carried out at the Clinical Hospital, Ljubljana, Slovenia, during 1925 to 1949. These data are shown in Table IV.

TABLE IV.—*Cases of Cancer of the Lung Among Autopsies Carried Out at the Clinical Hospital, Ljubljana, 1925 to 1949.*

Period.	Number of autopsies.								
	All cases.			All cancer.			Cancer of lung.		
	Male.	Female.	Total.	Male.	Female.	Total.	Male.	Female.	Total.
1925–9	564	553	1117	72	65	137	8	2	10
1930–4	793	765	1558	117	91	208	5	1	6
1935–9	1063	868	1931	132	113	245	11	1	12
1940–4	1080	1050	2130	129	135	264	18	1	19
1945–9	1929	1589	3518	246	292	538	28	9	38
1948–9	*897*	*791*	*1688*	*141*	*154*	*295*	*19*	*7*	*26*

See footnote to Table III.

Switzerland.

(1) We are indebted to the Bureau Fédéral de Statistique for information about the amount of tobacco consumed over the period 1936 to 1949, and to the Direction Général des Douanes for information about the sources of imported tobacco. The data are summarized in Table V and Fig. 1.

TABLE V.—*Consumption and Sources of Tobacco in Switzerland.* 1936 *to* 1949.

Year.	Tobacco consumption (lb. per head).	Percentage of tobacco			
		Home-grown.	Imported from		
			Balkans.	U.S.A.	Other areas.*
1936	3·5	6	10	38	46
1937	4·0	12	..	..	..
1940	4·6	12	..	..	..
1943	4·9	18	..	..	..
1945	5·9	20	..	..	..
1947	5·7	19	..	..	..
1949	4·5	18	11	42	28

* Mainly Brazil and the East Indies.

We have no detailed information about the manner in which the tobacco is smoked, but until World War II cigarettes formed only a small proportion of the whole (Table X).

(2) Prof. H. v. Meyenberg has provided a summary of the autopsies performed at the Pathological Institute, Zürich, during the period 1927 to 1941. Among 20,681 autopsies, there were 3584 cases of cancer and 276 cases of cancer of the lung. These figures are compared with those from other countries in Table IX.

(3) It is, however, not necessary to be dependent on hospital statistics for an estimate of the incidence of cancer of the lung in Switzerland, as vital statistics are available for the whole country. We are indebted to the Bureau Fédéral de Statistique, Berne, for a statement showing the deaths attributed to cancer of the bronchi and lungs, by sex, for each year 1929 to 1949 (Fig. 1) and also for data on age distribution of the population. The crude death rates, shown in Table X, in comparison with the rates from England and Wales and from Norway, have been calculated from population statistics provided by Stocks (personal communication).

Norway.

(1) Prof. Leiv Kreyberg, of the University of Oslo, has supplied figures for the production of tobacco (pipe, cigars and cigarettes) for each year 1930 to 1947 (Fig. 2, and for selected years in Table VI).

Mr. O. Jakobsen, of the Norwegian Embassy, writes that " Norway normally imports 85 per cent of her tobacco from America (Virginian tobacco), 9 per cent from the Middle East (Turkish tobacco) and 6 per cent from elsewhere." The imports are practically equal to the consumption, as the only exports are to the mercantile marine. Tobacco, except cigars, is almost all imported as such, and not as cigarettes. Many persons buy the cheaper pipe tobacco and make their own cigarettes, obtaining thus nearly twice as many for a given sum ; hence the

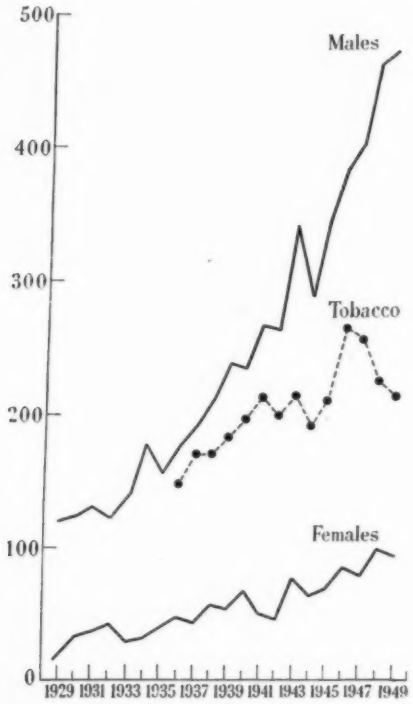

FIG. 1.—Cancer of lung. Deaths, 1929–1949. Consumption of tobacco (hundred thousand pounds), 1936–1949. Switzerland.

official figures for the consumption of pipe tobacco and cigarettes are misleading. Pipe-smoking is most common in rural areas.

TABLE VI.—*Consumption of Various Types of Tobacco in Norway.* 1931 *to* 1947 (*Kreyberg*).

Year.	Tobacco consumption (lb. per head).	Percentage of tobacco in form of:		
		Cigars.	Cigarettes.	Pipe tobacco.
1931	1·4	2	36	62
1932	1·4	3	35	62
1936	1·7	3	36	61
1937	1·9	3	39	58
1940	2·2	3	39	58
1943	1·0	1	49	49
1946	2·6	1	41	58
1947	2·9	1	44	55

(2) Prof. Leiv Kreyberg has supplied figures for the deaths attributed to cancer of the lung, by sex, for the years 1930 to 1948 (Fig. 2, 3), for deaths sepa-

rately in urban and rural areas, and for the population by sex. The calculated death rates from cancer of the lung are shown in Table X.

The national figures are reasonably reliable as, according to Mr. Jakobsen, "The doctor who has treated the deceased during his last illness is required to fill in an official certificate stating the cause of death . . . which must be stated in medical terms as precisely as possible, and it is not permitted to use general diagnoses such as "disease of the heart" or "disease of the lungs." The

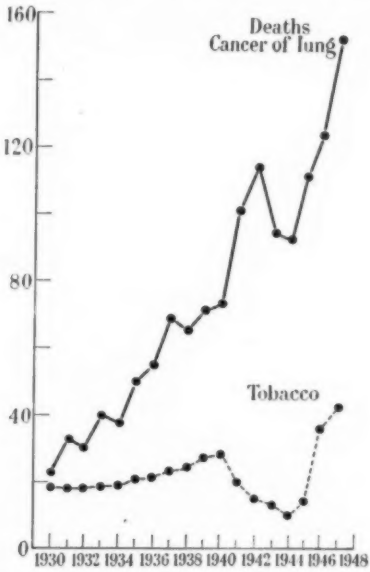

FIG. 2.—Cancer of lung. Deaths. Consumption of tobacco (hundred metric tons), Norway. 1930–1947. (Kreyberg.)

statement must be in accordance with an official nomenclature of causes of death. . . . The doctor is required to examine the patient after death."

(3) Twenty-eight per cent of the population were estimated to live in the cities of Norway in both 1930 and 1946; the proportion living in urban areas can, therefore, be considered to have been constant throughout the period under review. The following are the estimated crude death rates for each sex in urban and rural areas.

Norway

Death Rates of Cancer of Lung per million.

	Death rates of cancer of lung per million.					
	Urban.			Rural.		
	Male.	Female.	Persons.	Male.	Female.	Persons
1930–38	24	19	21	14	12	13
1939–47	63	42	52	30	24	27

The data show that :

(*a*) The incidence is greater in urban areas.

(*b*) The urban excess is slightly greater for men than for women—

1930–38 urban : rural ratio, male 1·7 : 1 ; female, 1·6 : 1.
1939–47 ,, ,, male 2·1 : 1, female, 1·8 : 1.

(*c*) The increase between 1930 and 1947 has been greater in the towns than in the country and greater for men than for women.

Urban increase male, 2·6 : 1 ; female, 2·2 : 1.
Rural ,, male, 2·1 : 1 ; female, 2·0 : 1.

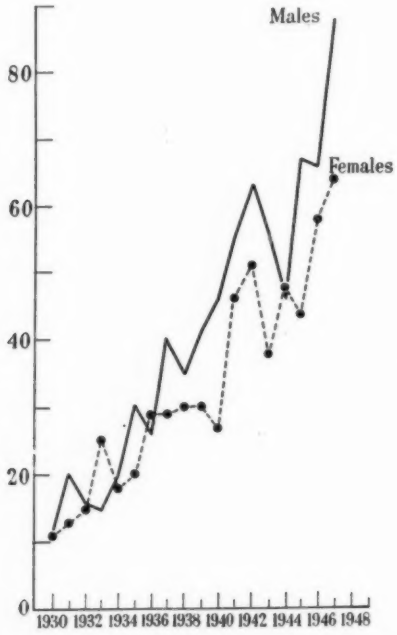

FIG. 3.—Cancer of lung. Deaths. Norway, 1930–1947. (Kreyberg.)

Sweden.

Snuff, used for chewing and for insufflation, makes up a very large but diminishing proportion of all the tobacco products consumed (Hutson, 1937 : Prof. Elis Berven, personal communication. Table VII). Prof. Berven writes, " Snuff is a very fine powder like the English snuff and is inhaled in small quantities through the nose. For chewing there are two types : (*a*) The same type of snuff (3–4 big pinches, i.e. about one teaspoonful, taken from the snuff-box) is put in between the lip and the mandible and is chewed backwards and forwards until it becomes a tough mass, in size of about a hazel-nut, which is lying at the same place about 3 to 4 hours ; . . . (*b*) Chewing tobacco is to be had in the form of braids,

TABLE VII.—*Distribution of Different Kinds of Tobacco as Per Cent of the Total Consumption. Sweden (Berven, personal communication).*

Year.	Cigars and cigar-cigarettes.	Cigarettes.	Tobacco for smoking.	Tobacco for chewing.	Snuff.	Tobacco.	Consumption (lb. per head per year.) Snuff.	Tobacco.
	%	%	%	%	%	%		
1908	10·8	3·3	4·4	7·9	73·6	26·4	2·08	0·7
1921	10·1	8·3	11·1	3·6	66·9	33·1	2·23	1·1
1931	9·3	16·1	13·0	1·8	59·8	40·2	1·73	1·2
1936	8·5	22·3	15·5	1·2	52·5	47·5	1·55	1·4
1944	6·7	27·9	23·7	0·6	41·1	58·9	1·15	1·7
1948	5·1	44·9	16·3	0·4	33·3	66·7	1·06	2·1
1949	4·7	46·9	15·6	0·4	32·4	67·6	1·02	2·1

from which they bite 1 to 2 cm. This piece is chewed to a half-firm consistence, which is then lying at the same place as the snuff." Unfortunately no figures for the incidence of cancer of the lung are available.

Iceland.

Smoking habits in Iceland are discussed by Dungal (1950), according to whom "The consumption of tobacco, particularly in the form of cigarettes, has certainly been much less in Iceland than in most European countries and America." Dungal's figures for cigarette consumption and for the incidence of lung carcinoma are summarized in Table IX.

Britain.

Data with regard to tobacco consumption and the incidence of cancer of the lung in Britain have been presented previously (Kennaway and Kennaway, 1947; Fig. 4, 5). New data on the autopsy incidence of cancer of the lung in British hospitals have been collected (Table VIII).

TABLE VIII.*—*Cases of Cancer of the Lung Among Autopsies Performed at Three British Hospitals,* 1925–48.

Hospital.	Period.	Number of autopsies. All cases. M.	F.	Total.	All cancers. M.	F.	Total.	Cancer of the lung. M.	F.	Total.
Glasgow. Western Infirmary (Cappell)	1925–9	..	..	1154	133	83	216	22	12	34
	1930–4	..	..	1214	153	82	235	31	5	36
	1935–9	..	..	1395	190	124	314	36	7	43
	1940–4	..	..	1273	180	124	304	41	16	57
	1945–8	..	..	1324	220	133	353	53	19	72
	1947–8	..	..	*687*	*103*	*68*	*171*	*31*	*9*	*40*
St. Mary's, London (Newcomb)	1930–4	827	481	1308	140	77	217	30	5	35
	1935–9	746	442	1188	171	112	283	36	9	45
	1940–4	..	..	..	..	..	..	..	..	..
	1945–8	683	428	1111	139	90	229	41	8	49
	1947–8	*371*	*229*	*600*	*83*	*47*	*130*	*23*	*6*	*29*
St. Bartholomew's, London (Cunningham)	1930–4	1056	558	1614	206	95	301	57	13	70
	1935–9	987	528	1515	192	121	313	37	10	47
	1940–4	..	..	..	..	..	..	..	..	..
	1945–8	544	217	761	111	60	171	43	10	53
	1947–8	*301*	*125*	*426*	*60*	*27*	*87*	*25*	*4*	*29*

* See also Table IX.

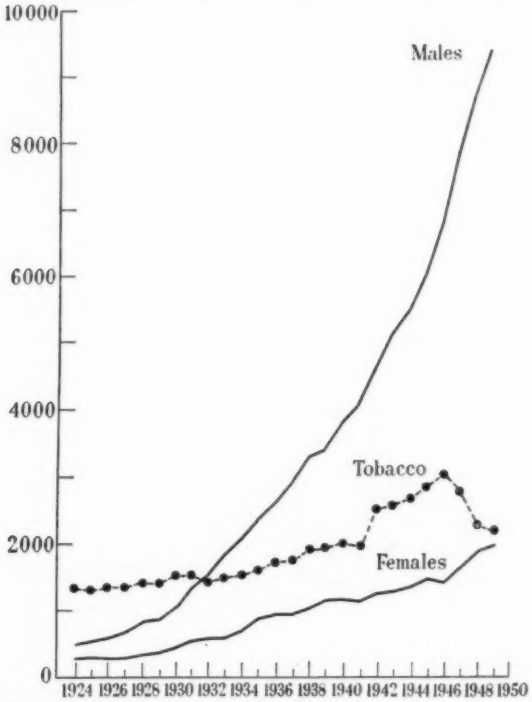

FIG. 4.—Cancer of lung. Deaths, England and Wales. Consumption of tobacco (hundred thousand pounds), United Kingdom, 1924–1949.

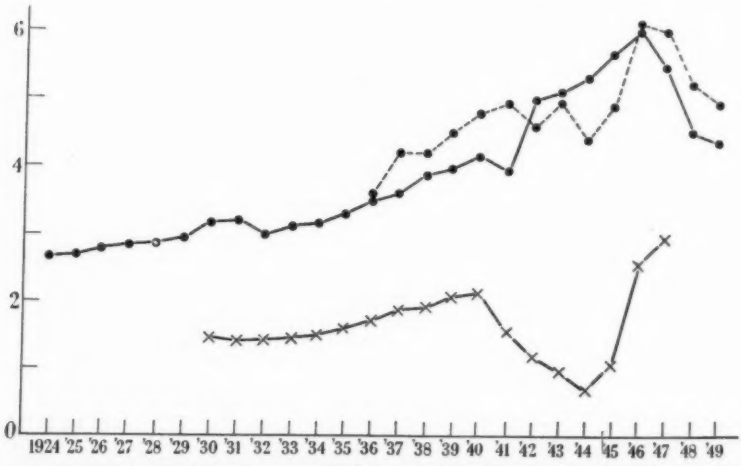

FIG. 5.—Consumption of tobacco (lb. per head). United Kingdom, Switzerland and Norway.

●——●——● United Kingdom.
●- - -●- - -● Switzerland.
×——×——× Norway.

Incidence of Lung Cancer.

Comparison of the data from various countries.

In Table IX hospital data from five sources in Britain and from Turkey, Yugoslavia, Switzerland and Iceland, are presented together. The cases of lung cancer are shown as percentages of the number of autopsies, and of the number of malignant growths found at autopsy; sexes are shown separately whenever the data are available. For comparison, estimations of the annual consumption of tobacco are included.

The data show that:

(1) A high proportion of autopsies on cases of cancer, between a fifth and a third, are now found to show cancer of the lung in British hospitals; 20 years ago the figure was between 10 and 15 per cent.

(2) The experience concerning males in Istanbul in recent years is not unlike the British experience.

(3) The experience in Slovenia is comparable with British experience in the first quarter of this century.

(4) The lowest recorded figures are found in Iceland.

(5) Swiss figures are comparable with Slovene ones. Swiss national statistics, however, indicate a higher incidence (Table X) and too much significance should not be attached to one hospital series.

Table X shows the crude death rates from cancer of the lung in England and Wales, Switzerland and Norway for selected years between 1931 and 1947, and estimates of the annual rates of consumption of tobacco.

[A comparison of the age-distribution in the three countries is given below. Mr. Olaf Jakobsen has kindly supplied figures for Norway by sexes for 1930 and 1946; the figures for the latter year are summarized in the table. The data for Switzerland were supplied by the Bureau Fédéral de Statistique, Berne.

Population.

Per cent of all ages.

	Males.				Females.		
	England and Wales, 1947.	Norway, 1946.	Switzerland, 1950.		England and Wales, 1947.	Norway, 1946.	Switzerland, 1950.
All	48·4	49·3	48·3	All	51·6	50·7	51·7
20–	7·4	8·6	7·4	20–	7·5	8·3	7·5
30–	7·8	7·9	7·2	30–	7·9	7·9	7·3
40–	7·1	6·6	7·1	40–	7·5	7·0	7·7
50–	5·3	5·0	5·3	50–	6·3	5·4	6·2
60–	4·0	3·3	3·7	60–	5·0	3·9	4·5
70–	2·6	2·7	2·4	70–	3·8	3·5	3·4

Males of the important age-group 50 to 69 make up a rather larger percentage of the population in England and Wales (9·3) than in Norway (8·3), while the position of Switzerland is intermediate (9·0); these small differences cannot account for the differences in the death-rates, and justify the use of crude death rates.]

TABLE IX.—*Cancer of Lung as Per cent of All Autopsies and of All Cancer Diagnosed at Autopsy in Different Countries and in Different Periods, Compared with Tobacco Consumption.*

Locality.	Period.	Cancer of the lung as per cent of: All autopsies. Male.	All autopsies. Female.	All autopsies. Persons.	All cancer autopsies. Male.	All cancer autopsies. Female.	All cancer autopsies. Persons.	Annual tobacco consumption (lb. per head).
Britain.								
16 British hospitals* (Passey and Holmes)	1894–1918	..	..	0·96–1·16	..	..	..	1900–02, 1·9 (cgts. 0·6)
	1919–1923	..	..	1·63	..	..	..	1906–08, 2·0 („ 0·7)
	1924–1928	..	..	2·11	..	..	..	1911–13, 2·1 („ 0·9)
								1923–25, 2·9 („ 1·8)
Leeds General Infirmary* (Bonser)	1891–1932	..	..	0·81–1·38	..	..	4·95–8·27	1929–31, 3·3 („ 2·4)
	1933–1937	..	..	2·31	..	..	11·32	1934–36, 3·6 („ 2·7)
Western Infirmary, Glasgow (Cappell)	1925–29	..	..	2·9	16·5	14·5	15·7	..
	1930–34	..	..	3·0	20·2	6·1	15·3	..
	1935–39	..	..	3·1	18·9	5·6	13·7	1936–37, 3·9 (cgts. 2·9)
	1940–44	..	..	4·4	22·8	12·9	18·8	..
	1945–48	..	..	5·4	24·1	14·3	20·4	1946–47, 5·0 („ 4·3)
St. Mary's, London (Newcomb)	1930–34	3·6	1·0	2·7	21·4	6·1	16·1	..
	1935–39	4·8	2·0	3·8	21·1	8·0	18·9	..
	1940–44	..	..	..	..	..	..	..
	1945–48	6·2	1·9	4·4	29·5	8·9	21·4	..
St. Bartholomew's, London (Cunningham)	1930–34	5·4	2·3	4·3	27·7	13·7	23·3	..
	1935–39	3·7	1·9	3·1	19·3	8·2	15·0	..
	1940–44	..	..	..	..	..	..	..
	1945–48	7·9	4·6	7·0	38·7	16·7	31·0	..
Turkey.								
Istanbul (Saglam, Schwartz)	1935–39	1·8	0·5	1·4	15·2	5·2	12·1	1937, 1·7
	1940–44	1·9	0·9	1·7	15·9	10·0	14·8	1942, 2·1
	1945–49	2·8	0·8	2·1	20·3	7·8	17·0	1947, 2·2
Yugoslavia.								
Ljubljana (Hribar, Kosir)	1925–29	1·4	0·4	0·9	11·1	3·1	7·3	..
	1930–34	0·6	0·1	0·4	4·3	1·1	2·9	..
	1935–39	1·0	0·1	0·6	8·3	0·9	4·8	..
	1940–44	1·7	0·1	0·9	14·0	0·7	7·2	..
	1945–49	1·5	0·6	1·1	11·8	3·1	7·1	..
Switzerland.								
Zürich (Meyenburg)	1927–41	..	..	1·33	..	..	7·7	1936, 3·5
Iceland.								
Reykjavik (Dungal)	1932–48	..	..	0·6	..	..	2·9	1913 (cgts.) 0·03 1932 („) 0·3 1947 („) 1·4
Britain.			Most recent period.					
Western Infirmary	1947–48	..	..	5·8	30·1	13·2	23·5	..
St. Mary's Hospital	1947–48	6·2	2·6	4·8	27·7	12·8	22·3	1947–48, 4·4 (cgts. 3·7)
St. Bartholomew's	1947–48	8·3	3·2	6·8	41·7	14·8	33·3	..
Turkey.								
Istanbul	1949–50	4·1	0·8	2·8	29.8	7·5	22·3	1949, 1·9
Yugoslavia.								
Ljubljana	1948–49	2·1	0·9	1·5	13·5	4·5	8·8	..

Yugoslavia.															
Ljubljana	.	.	.	1948–49	.	2·1	0·9	1·5	.	13·5	4·5	8·8	.	..	

TABLE X.—*Death Rates from Cancer of the Lung in England and Wales, Switzerland and Norway, Compared with Tobacco Consumption.*

Year.	England and Wales.*				Switzerland.				Norway.			
	Cancer of the lung. Death rates per million.			Annual tobacco consumption (lb./head).	Cancer of the lung. Death rates per million.			Annual tobacco consumption (lb./head).	Cancer of the lung. Death rates per million.			Annual tobacco consumption (lb./head).
	M.	F.	Persons.		M.	F.	Persons.		M.	F.	Persons.	
1931	85	31	57	3·2 (cgts. 2·3)	66	17	41	‡	14	9	12	1·4 (cgts. 0·5)
1932	93	32	61	3·3 („ 2·4)	62	19	40	‡	12	10	11	1·4 („ 0·5)
1936	142	43	91	3·8 („ 2·8)	86	21	52	3·5	18	20	19	1·7 („ 0·6)
1937	161	43	100	4·0 („ 3·0)	94	19	55	4·0 (cgts. 1939 1·4)	28	20	24	1·9 („ 0·7)
1940	222	55	131	†	115	31	71	4·6	31	18	24	2·2 („ 0·9)
1943	321	60	173	†	162	33	95	4·9	39	24	31	1·0† („ 0·5)
1946	366	71	206	5·4 (cgts. 4·6)	177	36	104	5·9	43	36	39	2·6 („ 1·1)
1947	398	78	228	4·5 („ 3·8)	183	34	106	5·7 (cgts. 1949 2·3)	56	40	48	2·9 („ 1·3)

* The figures available for tobacco refer to the United Kingdom, and those for cancer of the lung to England and Wales. If the smoking habits in Scotland and Northern Ireland are not very different from those in England and Wales, which contain 87 per cent of the population, this difference is of no importance.

† Disturbed by war.

‡ Not available.

Estimated population in thousands, Mid-year, 1947 (*Annual Abstract of Statistics*, No. 85 (1948).)

England and Wales	43,050	Great Britain, 48,189.
Scotland	5,139	
Northern Ireland	1,350	
United Kingdom	49,539	

The data show that:

(1) The apparent incidence of cancer of the lung is increasing in both sexes in all three countries.

(2) The rate of increase in the 10 years 1936–7 to 1946–7 has been much the same in the three countries; in the five years 1931–2 to 1936–7 it was more rapid in Norway than in England and Wales, and was slowest in Switzerland.

Death Rate from Cancer of the Lung.

1936–7 as percentage of 1931–2.

	England and Wales.			Switzerland.			Norway.		
	M.	F.	Persons.	M.	F.	Persons.	M.	F.	Persons.
1931–2	100	100	100	100	100	100	100	100	100
1936–7	170	137	162	141	111	132	177	211	187

1946–7 as percentage of 1936–7.

1936–7	100	100	100	100	100	100	100	100	100
1946–7	252	173	227	200	175	196	215	190	202

(3) The rate of increase has been greater for males than for females in England and Wales and in Switzerland.

(4) The incidence is comparatively low in Norway. In 1947 the crude death rate was approximately the same as it had been in England and Wales and in Switzerland 16 years previously.

	Population (both sexes) producing one death from cancer of the lung.	
	1931.	1947.
Norway	83,300	20,800
Switzerland	24,400	9,400
England and Wales	17,500	4,400

(5) The difference is essentially a difference of incidence in men. The incidence in women is similar in Norway and Switzerland and about twice as great in England and Wales (Table X).

The incidence of cancer of the lung is much more nearly equal in the two sexes in Norway than it is in the other countries which have been studied.

	Death rate from cancer of the lung in women as percentage of the death rate in men (1947).
Norway	71%
England and Wales	20%
Switzerland	19%

The ratios of cancer of the lung in women to total autopsies in women also indicates a relatively low incidence in women in Turkey and Yugoslavia (Table IX).

The relation between increases in the consumption of tobacco, and in cancer of the lung.

One should perhaps refer again, though there may be no need to do so, to the absence of any necessary connection between two quantities which are varying in the same way ; thus wireless licences in this country have multiplied at a rate very similar to that shown by cancer of the lung (Kennaway and Kennaway, 1947).

In Tables XI and XII comparisons are attempted of data from this country, Norway, and Switzerland during three periods, 1931–2, 1936–7 and 1946–7.

TABLE XI.—*Comparison between Tobacco Consumption, Cigarette Consumption and Death Rate (Persons) from Cancer of the Lung at Different Periods.*

Country.	Tobacco consumption (lb. per head).	Cigarette consumption (lb. per head).	Death rate from cancer of the lung.
1946–7 as percentage of 1936–7.			
England and Wales	128	145	227
Switzerland	155	170*	196
Norway	153	185	202
1936–7 as percentage of 1931–2.			
England and Wales	120	123	162
Norway	129	130	187

* 1949 as percentage of 1939.

In all three countries the increase in cancer of the lung was greater than the increase in tobacco consumption between 1931–2 and 1936–7, and between 1936–7 and 1946–7. The increase in consumption of cigarettes was more like the increase in lung cancer over the later period, but there was a considerable gap between the figures in England and Wales.

TABLE XII.—*Comparison between Tobacco Consumption, Cigarette Consumption and Death Rate (Persons) from Cancer of the Lung in England and Wales and Norway, and in England and Wales and Switzerland.*

Period.	Tobacco consumption (lb. per head).	Cigarette consumption (lb. per head).	Death rate from cancer of the lung.
England and Wales as percentage of Norway.			
1946–7	180	350	499
1936–7	190	446	444
1931–2	232	470	513
England and Wales as percentage of Switzerland.			
1946–7	085	162 (1949)	205
1936–7	104	266 (1939)	179

The difference in death rates between England and Wales and Norway was also very much greater than the difference in tobacco consumption ; it was,

however, reasonably close to the difference in cigarette consumption. In Switzerland tobacco consumption was much the same as in England and Wales, but death rates were only half as high. Differences in cigarette consumption again agree with the differences in death rates better than do differences in total tobacco consumption. This is because a much smaller proportion of the tobacco is consumed in the form of cigarettes in Switzerland than in England.

Clearly no exact proportionality has been found in the data so far available between the amount of tobacco smoked and the prevalence of cancer of the lung in different countries and at different periods. Cancer of the lung appears to increase more rapidly than does the use of tobacco ; such a change in effect may occur at a certain level in the dosage of a drug. A complicating factor is that when the population of a country consumes more tobacco, one does not know how much of this is due to the initiation of new smokers and how much to the increased use of tobacco by those who smoke already.

DISCUSSION.

(1) The high proportion of cancer of the lung found among autopsies on cases of malignant disease in males in Istanbul suffices to show that the arsenic content of tobacco has not provided any simple and exclusive explanation of the association between cigarette smoking and this form of cancer. It is perhaps too soon to say that arsenic is quite unobjectionable in tobacco, for we have no information about the minimum effective dose of any carcinogen in man, nor about the possible summation of effect of different carcinogens.

As yet we have autopsy figures only from a single centre in Turkey, and of course more data from this and other sources, in Greece and Bulgaria, for instance, are very desirable. The figures from a single hospital in Yugoslavia suggest an incidence of cancer of the lung comparable to the British figures of 30 years ago. We have no figures for the incidence of cancer of the lung upon the whole population of any East European country. The official figures for the consumption of home-grown tobacco are liable to the error, that a portion may escape record.

(2) We do not know whether tobacco smoke itself contains some carcinogen, or whether the practice of smoking renders the respiratory tract more susceptible to agents from external (e.g. the benzpyrene of coal-smoke) or internal, sources. If the carcinogen is in the tobacco smoke, and is in particulate form, we facilitate its penetration to the bronchi by inhalation through the mouth, thus evading the nasal filter. The evidence available at the moment is against the direct carcinogenic importance of tobacco smoke, because it tends to exclude the two most obvious carcinogens, arsenic and benzpyrene, which one might expect to be present. But the range of chemical structure of known carcinogens is now so wide that one must consider other possibilities.

(3) All the older data about cancer of the lung must be reviewed in the light of this connection with smoking ; one must reconsider the inverse relationship with sunlight (Stocks, 1947) and the very low incidence upon a rather curious assortment of occupations, namely, agriculture, coal-mining, and mule-spinning (Kennaway and Kennaway, 1947). The coal-miner who works below-ground cannot smoke during one-third of the day, and the mule-spinner, and those in many other occupations, cannot smoke while at work.

(4) The incidence of cancer of the lung in Switzerland, and in Istanbul, where comparatively little coal-smoke would be expected, is against the importance of

of this factor, but in this country (Stocks, 1936; Kennaway and Kennaway, 1947) and in Norway (see above) the mortality is higher in towns than in the country. In all such comparisons one must consider at least three possible factors, namely, (*a*) smoking habits, (*b*) facilities for diagnosis and treatment, and (*c*) atmospheric pollution with products of the combustion of coal tar, or of the internal combustion engine.

(5) The indication that cigarettes are more active than cigars and pipe tobacco in relation to cancer of the lung raises the question, whether this difference is due (*a*) to the method of combustion, or (*b*) to some property of cigarette tobacco. One cannot answer this question at present, but it is especially important in countries where smokers buy the cheaper pipe tobacco to make their own cigarettes. In Norway one obtains in this way nearly twice as many cigarettes for a given sum (Jakobsen, personal communication). One must consider three possibilities: (1) Pipe tobacco smoked in pipes; (2) pipe tobacco smoked in cigarettes; (3) cigarette tobacco smoked in cigarettes.

(6) The data given and discussed above from this country, Norway and Switzerland show that the study of the relations of national consumption of tobacco, and national incidence of cancer of the lung, has scarcely begun.

SUMMARY.

(1) Estimations of arsenic in cigarettes from the United States, Canada, England, Norway, France, Switzerland, Italy, Austria, Poland and Bulgaria show on the whole a transition from the arsenic-rich American type in the West, to the arsenic-poor Turkish type in the East; the latter is, of course, smoked in Western countries also.

(2) In Yugoslavia, Turkey, Greece and Bulgaria, the tobacco consumed is almost wholly home-grown, of Turkish type, and in the form of cigarettes.

(3) The high incidence of cancer of the lung at autopsy in one centre (Istanbul), in a country where Turkish tobacco is smoked almost exclusively, shows that the arsenic content of tobacco has not provided any simple and exclusive explanation of the association between cigarette smoking and this form of cancer. Cancer of the lung appears to be much less frequent at a centre in Yugoslavia (Ljubljana) than at Istanbul. More information from these and other Balkan countries is very desirable.

(4) Norway imports about 85 per cent of the tobacco consumed from the U.S.A., and 9 per cent from the Balkan countries. The incidence of cancer of the lung upon the two sexes is not very different (death rate, male to female, 1 : 0·7); it is greater in urban than in rural districts, and this difference is greater in men than in women. The increase in mortality in the last 20 years has been greater in men than in women, and greater in the towns than in the country.

(5) In Sweden, snuff makes up a much larger fraction (one-third in 1949) of the total tobacco products consumed than is recorded in other countries.

(6) A comparison is made of the data available for the increases since 1931 in (*a*) deaths attributed to cancer of the lung, and (*b*) in the consumption of tobacco, in England and Wales, Norway and Switzerland. The consumption of tobacco per head has been for the last 10 years rather higher in Switzerland than in the United Kingdom, and in Norway has been about one-half that in the other two countries, while the crude death rates at the beginning and end of the period were

roughly in the proportion of 10 (England and Wales) to 5 (Switzerland), and 2 (Norway). Cigarette consumption was approximately in the proportion of 4 (England and Wales) to 2 (Switzerland) and 1 (Norway) and was more in accord with the relative death rates. The increase in the number of deaths has been about the same (twofold) in all three countries, but the increase in consumption of tobacco and cigarettes has been less. The differences in the incidence of cancer of the lung are therefore quite different in extent from those in the quantity of tobacco consumed ; they are more like (though still different from) those in the quantity of cigarettes consumed. The study of the relation between the national consumption of tobacco and the national incidence of cancer of the lung has scarcely begun.

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REFERENCES.

BRADFORD-HILL, A., AND FANING, E. L.—(1948) *Brit. J. indust. Med.*, **5**, 2.
Chief Inspector of Factories and Workshops.—(1939) *Ann. Rep.*, p. 22. London (H.M. Stationery Office).—(1943) *Ibid.*, p. 45.
CURRIE, A. N.—(1947) *Brit. med. Bull.*, **4**, 402.
DAFF, M. E., AND KENNAWAY, E. L.—(1950) *Brit. J. Cancer*, **4**, 173.
DOLL, R., AND BRADFORD-HILL, A.—(1950) *Brit. med. J.*, ii, 739
DUNGAL, N.—(1950) *Lancet*, ii, 245.
HEADY, J. A., AND KENNAWAY, E. L.—(1949) *Brit. J. Cancer*, **3**, 311.
HENRY, S. A.—(1950) *Ann. Roy. Coll. Surg. England*, **7**, 425.
HUTSON, J. B.—(1937) *U.S. Dept. of Agric. Technical Bulletin No.* 587.
KENNAWAY, E. L., AND KENNAWAY, N. M.—(1947) *Brit. J. Cancer*, **1**, 260.
MONTGOMERY, H., AND WAISMAN, M.—(1941) *J. Invest. Derm.*, **4**, 365.
NEUBAUER, O.—(1947) *Brit. J. Cancer*, **1**, 192.
SAGLAM, T.—(1944) *Bull. Fac. Med. Istanbul.* Yil : 7, Sayi : 2, Imumi No. **28**, Nisan-Haziran, 1944, p. 3793.
SEMON, H. C.—(1945) *Proc. Roy. Soc. Med.*, **38**, Section of Dermatology, 128.
STOCKS, P.—(1936) *Ann. Rep. Brit. Emp. Cancer Camp.*, **13**, 239.—(1947) ' Studies on Medical and Population Subjects No. 1. Regional and Local Differences in Cancer Death Rates.' London (H.M. Stationery Office).
WYNDER, E. L., AND GRAHAM, E. A.—(1950) *J. Amer. med. Ass.*, **143**, 329.

THE AGE CURVE IN LUNG CANCER.

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MOST curves indicating the cancer death rates per million living in the different age-groups rise continuously with advancing age. The steepness of the rise generally falls off with increasing age, and in some cases in the oldest age-group it changes to a decline. This not only holds true for the curves for the total cancer mortality, but also for those for mortality from cancer of the various organs.

Not so in lung cancer. In 1949, in the Netherlands, the age curve for lung cancer in males began to decline at an age lower than is ever seen in other cancers. In 1945, in England and Wales, this exception to the rule was still more prominent

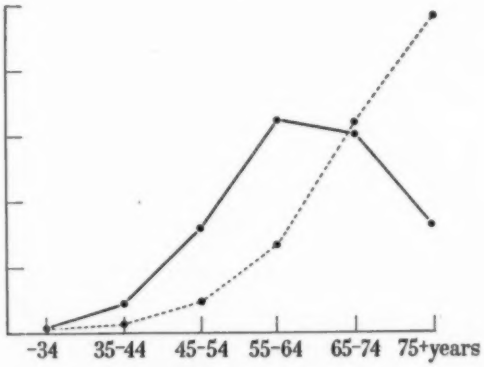

FIG. 1.—Age curves for cancer in males, England and Wales, 1945. Note that in order to make comparison easier, the age curves in Fig. 1 and 5 are given on a different scale. First, the death rates per million living of the six age-groups were determined. Then the sum totals of these numbers were reduced to 100 and a proportional reduction was made for the numbers of each age-group separately. The length of the ordinate for a given age-group, therefore, gives the percentage pertaining to that age-group of the sum of the lengths of the ordinates of all age-groups together.

——— Lung cancer (5941 cases).
. . . . . Cancer, all sites, lung cancer excepted (30,835 cases).

(Fig. 1). The numbers of lung cancer deaths in the Netherlands and in England and Wales, 1125 and 5941 respectively, were large enough to exclude the possibility that these exceptions to the rule might be caused by chance only.

Is this decrease of the cancer death rate at a relatively early age a characteristic of lung cancer or is there some other explanation?

Dormanns (1936) suggested that it might be caused by the increase of lung cancer in the course of the last 30 years, for, if the irritative factors responsible for lung cancer exercise their influence especially in younger people, the death rate among these may already increase at a moment when in the older age-groups their influence is not yet perceptible to the same degree.

Until recently it was impossible to prove which of these suppositions was right. Table LXXX of the *Statistical Review of England and Wales for 1940–1945* (Registrar-General, 1949), part of which is here reproduced as Table I, offers us the key to the solution of this problem.

TABLE I.—*Lung Cancer in Males. Death Rates per Million Living by Age*, 1911 *to* 1945.

Year.	0–34.*.	35–.	45–.	55–.	65–.	75+.
1911–20	3	13	34	64	76	42
1921–30	3	29	73	128	136	92
1931–35	5	69	217	350	347	239
1936–39	8	99	335	579	570	381
1940–44	8	129	453	866	799	498
1945	10	144	555	1116	1050	571

* Equivalent average death rate.

In Fig. 2, which is derived from Table I, the age curves for males in all year-groups given show the same early decline of the lung cancer death rate, with the

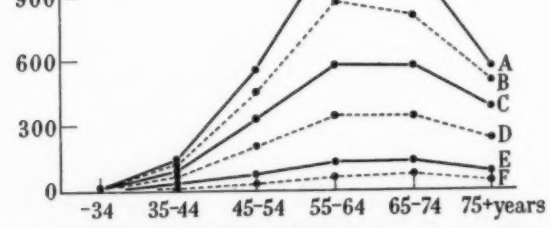

FIG. 2.—Lung cancer in England and Wales. Males. Death rates per million living by age. A, in 1945; B, 1940 to 1944; C, 1936 to 1939; D, 1931 to 1935; E, 1921 to 1930; F, 1911 to 1920.

only difference that in 1911 to 1930 the falling off began at a somewhat older age than in the later years. These age curves, which in Table I follow one another horizontally, express the increase of the tendency to cancer with increasing age.

The curves in Fig. 3, constructed from the numbers following one another vertically, show us, for each age-group separately, the increasing lung cancer mortality in the course of years. Among those under 35 years of age lung cancer has not increased greatly. In that age-group the number of faulty diagnoses must be very large, especially where young children are concerned. With advanc-

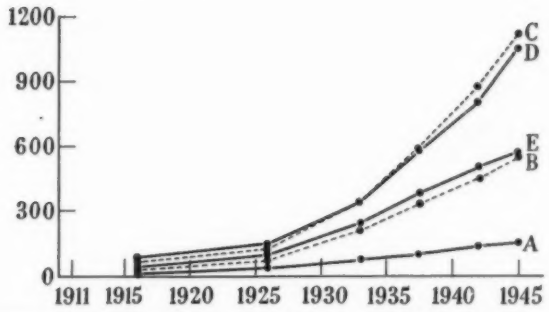

FIG. 3.—Lung cancer in England and Wales. Males. Increase in different age groups per million living from 1911 to 1945. A, 35 to 44; B, 45 to 54; C, 55 to 64; D, 65 to 74; E, 75 and over.

ing age also the diagnosis will often be wrong, but I will not go into this problem, as it is only a few years since Kennaway and Kennaway (1947) discussed it in detail. In all the older age-groups the increase has been large and continuous, and even in 1945 it showed no signs of slowing down. From this we conclude that in the years to come the death rate in each age-group will go on increasing.

From the curves given in Fig. 3 we can derive, by interpolation, for each year and each age-group separately the approximate values of the cancer death rates. This has been done for the years 1915, 1925 and 1935. The figures found in this

TABLE II.—*Lung Cancer in Males. Death Rates per Million Living by Age* 1915, 1925, 1935, and 1945.

Year.	0–34.	35–.	45–.	55–.	65–.	75+.
1915	3	12	32	55	75	40
1925	3	29	75	125	140	90
1935	6	85	275	460	455	312
1945	10	144	555	1116	1050	571

way, in combination with those already known for 1945, are given in Table II. Fig. 4 gives the age curves constructed from these figures.

For a full understanding of the meaning of Fig. 4 it is necessary to take the following points into consideration: Cancer only develops after a long period of preparation, which in most cases can be counted in decades. Those who at the age of 80 contract cancer, do so partly because of a tendency to cancer years before—let us say at the age of 70. They are recruited from the group of the younger ones, whose tendency to cancer is also known, as it is given by the length of the ordinate belonging to that age in Fig. 4. It would be wrong, therefore, to compare in this respect persons who in 1945 were 80 years of age with persons who, in the same year, were 70 years of age. Surely this comparison should be made with those who, ten years earlier, in 1935, belonged to that age-group. And again, the tendency to cancer among these last-named should be compared with that of those who, in 1925, were 60 years of age.

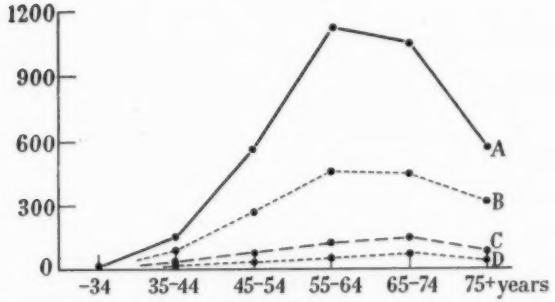

FIG. 4.—Lung cancer in England and Wales. Males. Death rates per million living by age. A, in 1945; B, in 1935; C, in 1925; D, in 1915.

Fig. 5 gives the lung cancer age curve constructed according to this principle. It does not differ in any way from all the other cancer age curves. This is evident by comparison with the age curve for " cancer all sites, lung cancer excepted " for males.

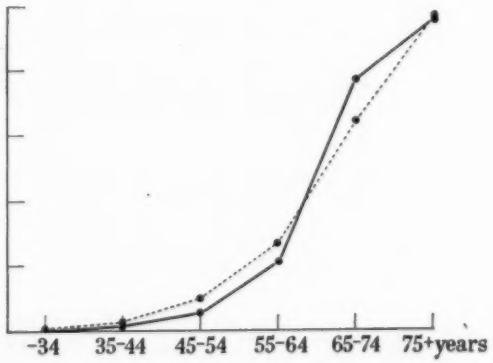

FIG. 5.—Age curves for cancer in males, England and Wales, 1945.
——— Lung cancer, after correction.
. . . . . Cancer all sites, lung cancer excepted. See note to Fig. 1.

From this investigation it appears that the so-called age curve for lung cancer in males with its decline at a relatively early age is nothing but an illusion. In fact the same objection can be made to a greater or less extent to all other age curves constructed in this way. It is only by chance that most of these do not diverge far from reality, as most cancer death rates have not altered much in the course of the last decades.

DISCUSSION.

Various points require consideration.

First: Is not this reasoning in conflict with the fact that in the periods 1911 to 1930 the lung cancer age curve showed almost the same characteristics

as in 1945 ? Almost, I write, as there was a difference, as is clearly visible in Fig. 4. In my opinion this objection is not valid, as an increase from 5 to 10 is of the same order as an increase from 100 to 200. Evidently lung cancer is a product of this century. Previously its occurrence was the greatest exception.

Second : I made the supposition that the irritative factors promoting lung cancer exercise their influence especially in younger people. If it were only persons of, let us say, from 25 to 45 years of age who were exposed to these factors, then it would be incomprehensible that already 20 years ago those belonging to the higher age-groups also showed such a considerable rise in the lung cancer death rate. Besides, if it were only these younger people who are exposed to the irritative factors, then it would be conceivable, indeed, that the steepness of the age curve would fall off at an older age, but it would be inconceivable that this would have led to such a sharp decline. We must presume, therefore, that it is not only the younger people who are endangered by these irritative factors.

Third : Let us suppose that all males were exposed to these irritative factors to about the same degree, and that in each of the last 40 years the quantity of these factors had increased considerably. Were this so, then really we would expect to find an " age curve " for lung cancer approximately such as we did find. If one fact proves the reality of the great increase of lung cancer in the last 40 years, it is the shape of its " age curve," now demonstrated as being an illusion only.

Artificially, with older age, the lung cancer age curve is pushed down. If, at this moment, the increase of lung cancer in the younger age-groups came to a halt, even then it would progress in the older age-groups up till the moment when the illusionary age curve changed into the real one. We can put it in another way : At this moment the lung cancer mortality is lower than corresponds with the total amount of the causative irritative factors. The lung cancer " ceiling " is lying at a far higher level than the mortality figures would suggest.

Up to the age-group 55 to 64 inclusive the shape of the illusionary age curve is about normal ; it is only after that, that the sharp decline begins. In 1945, in England and Wales, in males less than 65 years of age, the number of deaths from lung cancer was 33·8 per cent of those from " all cancers with the exclusion of lung cancer " (Registrar-General, 1947). By analogy with what we found just now, it can be assumed that, if the lung cancer age curve in 1945 had its real shape, the number of all deaths from lung cancer would have been 33·8 per cent of those from " all cancers with the exclusion of lung cancer, " namely, $\frac{33{\cdot}8}{100} \times 30{,}835 = 10{,}422$, which is 4481 more than in reality died from lung cancer. In 1945, therefore, the " ceiling " of lung cancer mortality in males corresponded with a level of 10,422 (Fig. 6). This also means that in this case the total number of cancer deaths in males would have been $30{,}835 + 10{,}422 = 41{,}257$, and not 36,776, which was the number given in the *Statistical Review* for 1945 (Registrar-General, 1947). Following the same reasoning it appears that in 1949, in the Netherlands, for males this " ceiling " was lying at a level of 1710, whereas the lung cancer deaths registered numbered 1125 only.

It is clear that the " ceiling " will never be reached except after a long period of rest, with stationary lung cancer death rates in the younger age-groups. We are still far from such a period of rest, as is clearly demonstrated by the sharp increase of lung cancer between 1945 and 1948. In 1948, in England and Wales,

the number of deaths from lung cancer in males was about 8282.* The age curve was practically the same as in 1945. In 1948, therefore, the " ceiling " for lung cancer was lying at a level of 14,640, so that there was a deficit of 6,358. If in 1948 the " ceiling " had been reached, the crude death rate at all ages per million living in males would have been approximately 2277 for " cancer all sites " and 734 for " cancer of lung and mediastinum," as against 1960 and 422 respectively, the rates actually reported.

Table III gives, for England and Wales, for the years 1939 to 1948, the absolute numbers of deaths in males and females for " cancer of lung, pleura and mediastinum " and for " cancer all sites with the exclusion of the first-named organs."

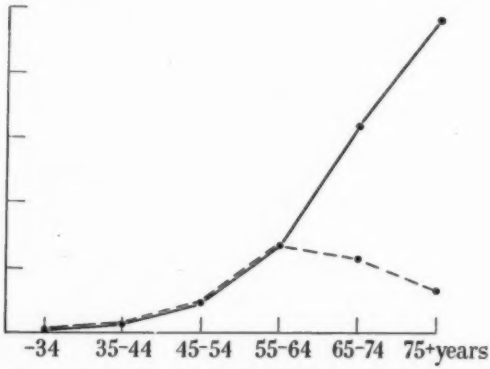

FIG. 6.—Balance of lung cancer mortality in males, England and Wales, 1945:

Lung cancer deaths recorded	5941
Deficit	4481
Lung cancer risk	10,422

——— Lung cancer age curve after correction.
. . . . . Pseudo lung cancer age curve.

After having studied this table we no longer wonder that in England and Wales cancer in males is again on the increase. Lung cancer accounts for the greater part of this. And still, if in 1948 the " ceiling " of lung cancer had been reached, the total number of cancer deaths would not have been 40,026, but more than 46,000 !

In females lung cancer is also increasing. An analysis of its age curve, such as I have made in this paper for lung cancer in males, shows us that, just as for males, the real age curve for females closely resembles that for the " non-hormonal cancers " (cancers other than those of breast and uterus) in females.

The problem which lung cancer presents to us is a very serious one, not only for England and Wales, but also for the Netherlands and many other countries.

* In the *Review* for 1948 (Registrar-General, 1950) the numbers for lung cancer are only given in combination with those for cancer of the mediastinum. During the last 40 years cancer of the mediastinum has not changed much in frequency. In 1945, 289 cases of mediastinal cancer were recorded, as against 5941 cancers of the lung. For the following computation I have assumed that in 1948 the number of mediastinal tumours was the same as in 1940, namely, 315, and that the distribution over the different age-groups in both years was the same.

TABLE III.—*Deaths from Cancer for Both Sexes at all Ages. England and Wales, 1939 to 1948.*

Year.	Males.		Females.	
	Lung, pleura, mediastinum.	All sites, lung and pleura and mediastinum excluded.	Lung, pleura, mediastinum.	All sites, lung and pleura and mediastinum excluded.
1939	3751	28,326	1149	33,907
1940	4047	29,088	1180	34,425
1941	4260	29,226	1155	34,333
1942	4758	29,253	1266	34,862
1943	5236	29,716	1294	35,568
1944	5610	29,271	1358	35,449
1945	6076	30,182	1477	36,018
1946	6818	30,302	1556	36,731
1947	7805	30,943	1730	37,171
1948	8597	31,429	1866	37,540

We are now able to realize how great this danger is, and it seems time to draw the conclusions from it and to accept their consequences.

SUMMARY AND CONCLUSIONS.

A study of the statistics of lung cancer reveals the fact that the so-called lung cancer age curve with a sharp decline at a relatively early age is incorrectly considered as such. For males as well as for females the lung cancer age curves have the same shape as those for all the other irritational cancers. Their decline with older age is the consequence of the extraordinary increase in the amount of environmental factors promoting lung cancer.

If at this moment the danger offered by these environmental factors became stationary, it would be more than likely that even then the number of lung cancer deaths would go on increasing up to about double the present figure. The threat of lung cancer is so great that nothing should be left undone which might lead to the prevention of this disease.

I wish to express my indebtedness to Prof. E. L. Kennaway (1950) for drawing attention to the most valuable material contained in the publications of the General Register Office of England and Wales, and to the Registrar-General of the Netherlands for placing at my disposal material, some of which is as yet unpublished.

REFERENCES.

DORMANNS, E.—(1936) *Report of Second International Congress against Cancer*, **1**, 460.
KENNAWAY, E. L.—(1950) *Brit. J. Cancer*, **4**, 158.
Idem AND KENNAWAY, N. M.—(1947) *Ibid*, **1**, 260.
REGISTRAR-GENERAL.—(1947) 'Statistical Review of England and Wales for the year 1945.' London (H.M. Stationery Office).—(1949) 'Statistical Review of England and Wales for the Six Years 1940–1945.' London (H.M. Stationery Office).—(1950) 'Statistical Review of England and Wales for the Year 1948.' London (H.M. Stationery Office).

TRISETHYLENEIMINO-S-TRIAZINE IN HUMAN MALIGNANT DISEASE: A PRELIMINARY TRIAL.

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THE measure of success which has attended the use of the nitrogen mustards in the palliation of certain forms of human malignant disease, notably those of reticulo-endothelial origin, has led to a wider search among compounds of the same and of analogous chemical type for alternative and more efficient therapeutic agents.

The water-soluble trisethyleneimino-s-triazine (Fig. 1) has been investigated in this connection independently on both sides of the Atlantic. In England its

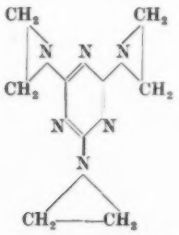

FIG. 1.—Trisethyleneimino-s-triazine.

cytotoxic and tumour-inhibitory activity were first demonstrated in the Research Laboratories of Imperial Chemical Industries Ltd. (Rose, Hendry and Walpole, 1950), where it is known as "9500."

The compound has been tested for its effect upon a wide variety of biological materials (Rose, Hendry and Walpole, 1950; Lewis and Crossley, 1950; Burchenal, Crossley, Stock and Rhoads, 1950; Burchenal, Johnston, Cremer, Webber and Stock, 1950). In our own laboratories it has been tested on cells grown *in vitro*, and upon leukaemia and tumours in rodents.

Effects on Animal Tissues and Tumours.

Effect on cells in vitro.

Chick fibroblasts which had been growing for 24 hours as hanging drop preparations were treated by the addition to the medium of a drop of 9500 in Tyrode solution, in concentrations ranging from 12·5 mg./litre to 62·5 mg./litre. At the end of 80 minutes the cultures were fixed and stained and mitotic counts were done. As a control similar sets of cultures were treated with Tyrode solution.

The reduction in mitosis is shown in Fig. 2, where it will be seen that the addition to the medium of the drug in a concentration of 60 mg./litre reduces mitosis to less than one half. With the technique used it is not possible to state the final concentration in the medium which would of course be less. This reduction in mitosis is quantitatively similar to that found 80 minutes after the application of 30 r X-rays.

The effects produced also demonstrate the direct action of the drug on cells.

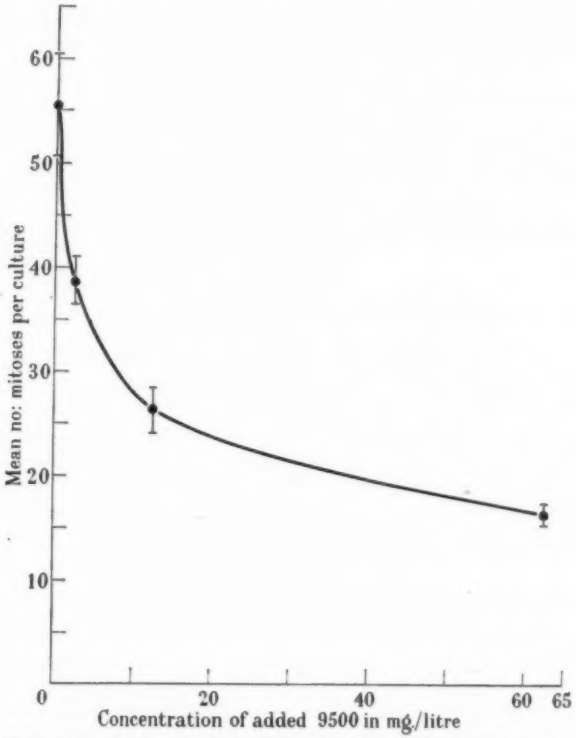

FIG. 2.—Reduction in mitosis in fibroblasts, fixed 80 minutes after the application of various concentrations of 9500.

Mouse leukaemia.

Mice of the Afb strain injected intraperitoneally with leukaemic cells of monocytic type from a spontaneously occurring case, were given the drug intraperitoneally in 5 γ doses, twice daily, starting 24 hours after the injection, for a period of 5 days; in one experiment a lengthening of survival time over several months was obtained. Re-treatment, at the same dose levels, lengthened survival time still further. In a second experiment, using leukaemic cells from a different mouse with lymphocytic leukaemia, the mean survival time was lengthened by only a few days.

Rat lymphosarcoma.

Rats of the American Wistar strain were injected with a rat lymphosarcoma subcutaneously. After 20 days eight of ten untreated animals had been disposed of on account of the size of the tumours. Two groups, each of ten rats, were given 9500 intraperitoneally in doses of 0·5 mg./kg. spread over 5 days and the effects assessed after 20 days. In one group treatment was started 6 days after the injection, when the tumour was established ; in this group four rats had been disposed of. In the second group in which treatment was started 24 hours after the injection of the tumour, only three rats had been disposed of, and in five no tumour appeared.

Mouse mammary adenocarcinoma.

Grafts of mammary carcinoma from the C3H and A strain of mice were established in pure-bred mice of the same strains. The drug was given intraperitoneally in doses of 5 γ twice daily over a 5-day period. There was no difference between treated and untreated mice in the time of disposal.

All experimental animals have shown a temporary loss of weight after being given the drug.

Effects on Human Malignant Disease.

This report, which is preliminary in nature, describes the testing of the compound in 17 patients suffering from leukaemia, polycythaemia vera, lymphadenoma, multiple myeloma and three types of carcinoma.

Cases were chosen because they were unfavourable or too advanced for other forms of treatment. This choice of the least favourable cases makes it harder to compare the effects of the drug with effects obtained with other forms of therapy. It is, however, of value to compare in any individual patient the effects of the drug against those of any other type of appropriate treatment given either prior or subsequent to 9500. This we have done where possible.

The drug was given intravenously in doses ranging from 0·09 mg./kg. to 0·22 mg./kg. the total dose usually being spread over 3 days. On the basis of our experience we feel that a total dose between 0·15 and 0·18 mg./kg. probably represents a working range within which toxic effects on the bone marrow are not serious.

Side effects were slight ; half the patients had anorexia or nausea, generally very slight. The only patient who vomited did so after a dose of 0·22 mg./kg., the highest dose we have given. The gastric side effects are therefore much less than those accompanying treatment with the nitrogen mustards. Furthermore, no local effects occur at the site of the injection ; in this respect 9500 has the advantage over the nitrogen mustards.

Leukaemia and polycythaemia.

Nine patients in all were treated ; of these one is excluded from analysis as death occurred from pneumonia shortly after completion of treatment, and following an anaesthetic for a coincidental prostatic obstruction. Of the six leukaemia cases reported, four were either terminal chronic cases or else acute in type. Transfusions were necessary in several of the patients before and after

treatment and this necessity has to some extent confused the assessment of the value of the drug. Some of the data on these cases are given in Table I.

TABLE I.

Number.	Sex.	Age.	Diagnosis.	Total dose. (mg./kg. over 3 days).	Side effects.
I	F.	50	Chronic myeloid leukaemia	0·22	Nausea and vomiting.
II	F.	42	Ditto	0·15	Nausea
III	F.	63	Myeloblastic leukaemia	0·15	*Nil*
IV	F.	61	,, ,,	0·22	Nausea
V	F.	62	Subacute lymphoid leukaemia	1st Course 0·17 2nd ,, 0·13 3rd ,, 0·18	*Nil*
VI	M.	3	Acute lymphoid leukaemia	1st Course 0·1 (1 day) 2nd Course 0·2 (2 days)	,,
VII	F.	74	Polycythaemia and leukaemia	0·15	,,
VIII	F.	66	Polycythaemia	1st Course 0·09 2nd ,, 0·12	Nausea

Case I. Chronic myeloid leukaemia.—This patient was in fair general condition and had previously been treated successfully with urethane, the remission so obtained lasting for one year. Considerable improvement in well-being followed treatment with 9500; her weight which was 45 kg. before treatment increased to 54 kg. during the 6 months she has been under observation. The splenic enlargement decreased within 2 weeks and this decrease, while not dramatic, was maintained. The dose used in this case resulted in a temporary fall in haemoglobin and a fall in the white blood count to 1000 per c.mm., at which time she was given a simple blood transfusion. The haemoglobin percentage recovered and 6 months later was above pre-treatment level. The percentage of primitive cells fell after treatment, but had reached the original level at 6 months (Fig. 3).

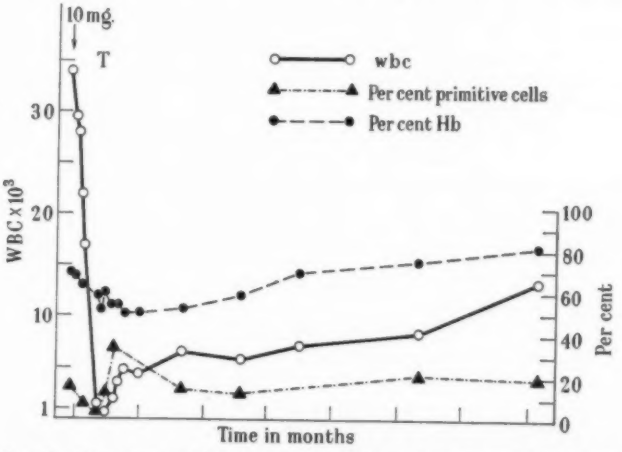

FIG. 3.—Case I: Chronic myeloid leukaemia. Effect of 9500 on peripheral blood count.

In this case the improvement obtained was about the same as would be expected after a non-repetitive course of treatment with X-rays or urethane.

Case II. Chronic myeloid leukaemia.—This patient had previously obtained a satisfactory remission of 14 months' duration with a single course of splenic irradiation. At the end of this remission she was suffering from lassitude and abdominal discomfort, and a rising white blood count. Following treatment with 9500 her well-being improved ; her white cell count fell and the haemoglobin, which was 86 per cent, rose slightly. Primitive cells fell from 26 per cent to 14 per cent within 4 days. The subsequent history of this patient was complicated, 2 months after treatment, by hepatogenous jaundice with leukopenia. Four months after treatment the haemoglobin began to fall and the white cell count commenced to rise, and sore throat appeared, primitive cells rising above the pre-treatment level. She was re-treated a month later with 9500, no beneficial effects being observed in the week that elapsed between treatment and death. Post-mortem examination showed a massive leukaemic infiltration of liver, kidneys and spleen. The bone-marrow from femur and sternum appeared active. Multiple capillary haemorrhages were present in the myocardium and brain. It is difficult to assess the effect of treatment in this case owing to the complicating factors. The remission, however, was not as satisfactory as that previously obtained with X-rays.

Case III. Myeloblastic leukaemia of Naegeli type.—This was a terminal case which had been treated previously only with transfusions. Within 7 days after treatment the haemoglobin rose and the white cell count fell from 145,000 to within normal limits. Primitive cells fell from a pre-treatment level of 76 per cent to 37 per cent and the myeloblasts and premyelocytes disappeared completely. However, this remission, although dramatic, was short. Two months later the patient was as ill as before treatment.

Case IV. Myeloblastic leukaemia.—This rather acute case showed a genuine improvement, although of short duration, following treatment. Prior to admission she had been transfused and it was necessary to repeat transfusions for the very low red cell count. An improvement in the leukaemic condition was prompt. Five days after the first treatment the total white cell count was within normal limits and immature cells had been reduced from 40 per cent to 6 per cent of the total. Improvement was not maintained and 3 months after treatment the immature cells had risen to pre-treatment level. At no time was the spleen palpable in this patient. The main findings are shown in the graph (Fig. 4), which demonstrates the immediate reduction in total white cells and the almost complete absence of circulating immature cells for some days following treatment. Incidentally it will be noted that transfusions did not reduce the percentage of immature cells.

Case V. Subacute lymphoid leukaemia.—This patient had been treated with splenic irradiation the response to which had lasted less than 2 months.

At the time of treatment with 9500 her general condition was extremely poor. The haemoglobin was 18 per cent and a high percentage of her white cells were immature. Transfusions were done repeatedly during treatment, but without any lasting effect on the red cell count. However, after each treatment with 9500 a dramatic fall in the white cell count occurred ; the immature cells, after an initial rise, fell markedly. The size of the spleen increased immediately after each treatment, then decreased in size. In this case the effects on total white

cells and on primitive cells seemed to be a result of the drug. The patient died 4 months after treatment with 9500. There was no post mortem examination.

Case VI. Acute lymphoid leukaemia.—This child presented with lymphosarcomatous tumours. The marrow was infiltrated with lymphocytes and these appeared in the blood stream in large numbers. There was no evidence that the drug affected the course of the disease.

Case VII. Polycythaemia vera.—This case of polycythaemia with leukaemia had been treated for 3 years with X-ray therapy ; over the first 2½ years splenic irradiation had been used, but as the remissions obtained became progressively slower and slighter in degree, whole-body irradiation had been instituted (175 r over 23 days). The resulting remission became apparent 2 months after treatment, but was reasonably satisfactory and lasted for about 4 months. She was

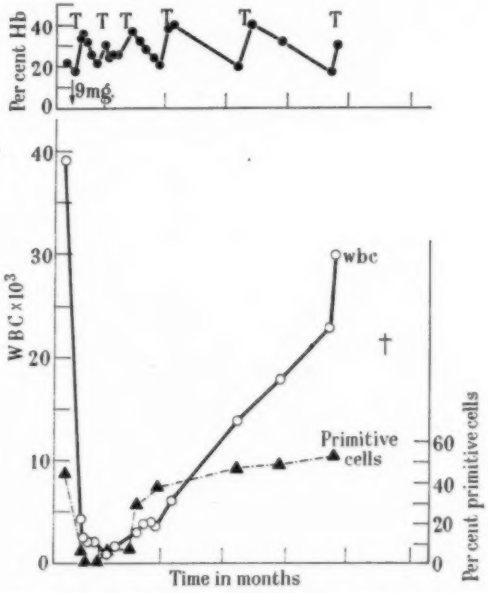

FIG. 4.—Case IV : Myeloblastic leukaemia. Effect of 9500 on peripheral blood count.

then given 9500. A good remission was noted 20 days after treatment, which compared well with her first and best remission following splenic irradiation. The patient felt better within a few days. A reduction in red cells and haemoglobin to normal levels was accompanied with a similar reduction in the leukaemic blood count. The platelet count remained high, but a difference in size was observed in that after treatment the platelets became larger. The spleen showed an immediate reduction in size.

Case VIII. Polycythaemia vera.—This patient had previously been treated with radio-active phosphorus, and as a consequence the red cell count and haemoglobin had been reduced to within normal limits. Very little effect was noted

on the enlarged spleen. The remission as far as the blood count was concerned lasted one month. After 9500 a similar reduction in red cells and haemoglobin occurred. This lasted 4 months, after which the symptoms recurred. The patient is feeble-minded and it is therefore not possible to assess the subjective improvement accurately.

In both polycythaemia cases the clinical effects were as good, or better, as had been obtained by their previous treatments. In neither patient, however, was a reduction in the platelets obtained.

Lymphadenoma.

In Table II are shown the doses and some of the constitutional and haematological results of the drug in Hodgkin's disease. The effects can be compared very exactly with those of the nitrogen mustards. Both drugs reduce the red cell count and haemoglobin and a temporary leukopenia generally occurs.

TABLE II.

Number.	Sex.	Age.	Diagnosis.	Total dose (mg./kg. over 3 days).	Side effects.	Hb.		W.B.C.	
						Fall in Hb (%)	Time of recovery.	Lowest count.	Time after treatment (days)
IX	M.	61	Generalized lymphadenoma	0·137	*Nil*	12	Unassessed; subsequent HN_2	No fall.	—
X	M.	67	Ditto	0·195	Slight nausea	4	Complete at 2 months	2100	14
XI	M.	40	„	0·18	*Nil*	None (transfusion)	—	2100	13
XII	F.	65	Multiple myeloma	0·15	Anorexia	16	Beginning 2 months	800	37
XIII	M.	48	Ditto	1st Course 0·10	„	12	Not recovered. 5 months	3300	3
				2nd Course 0·175	—	—	—	1100	21
XIV	M.	29	Seminoma	0·18	Nausea and malaise	12	Beginning 2 months	3600	10
XV	M.	66	Melanoma	1st Course 0·2	*Nil*	8	Not recovered. 2 months	3200	13
				2nd Course 0·2 (14 days)	—	—	—	2300	8
XVI	M.	64	Carcinoma alveolus	0·12 (7 days)	*Nil*	20 (bleeding from tumour)	—	No fall.	—

Case IX. Generalized lymphadenoma (section proved).—This patient was given a rather low dose of 9500. Nevertheless the enlarged nodes decreased very temporarily and the skin itching from which he suffered disappeared for about a week. The good effects were so transient that after an interval of 3 weeks he was given nitrogen mustard (0·4 mg./kg.). A longer lasting effect was obtained on his nodes and on his skin irritation. It is possible that a more comparable effect would have been obtained with 9500 at a somewhat higher dose.

B.C.

Time after treatment (days)
—
14
13
37
3
21
10
13
8
—

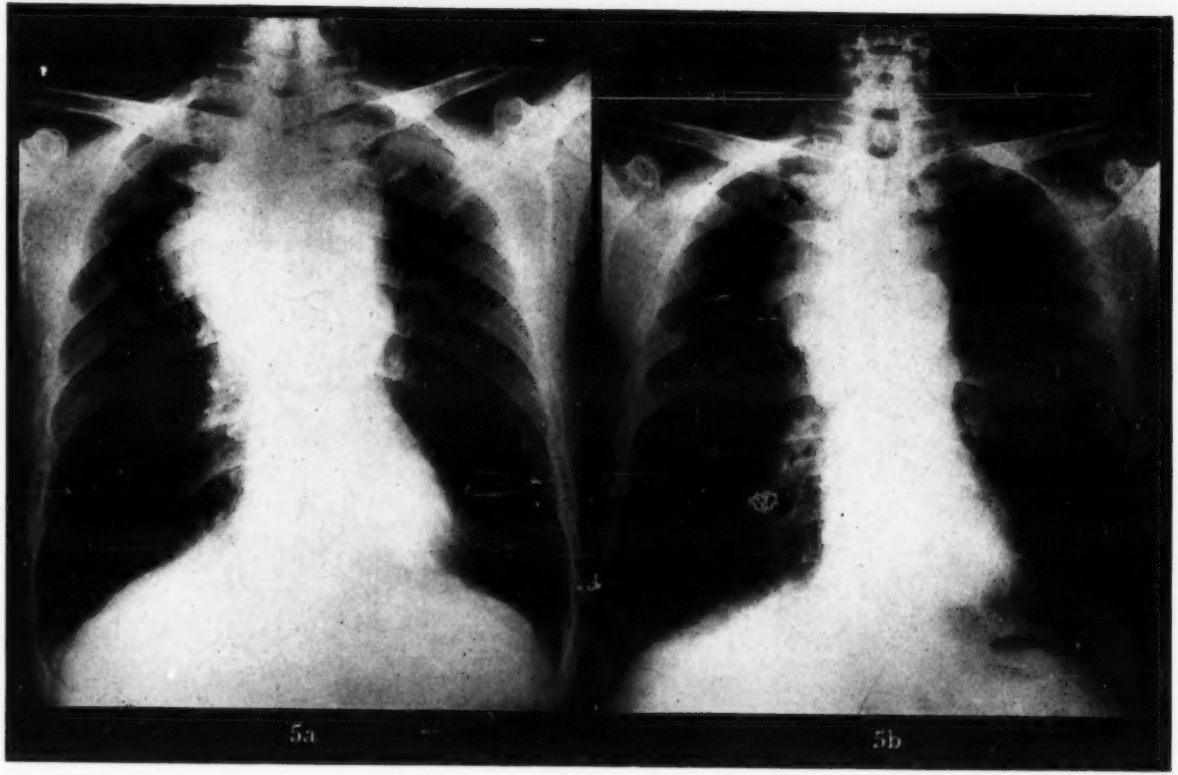

FIG. 5.—Case X : A. Mediastinal mass before treatment. B. Three weeks after treatment.

Paterson and Boland.

Case X. Generalized lymphadenoma.—The main symptoms in this case were severe skin itching and the presence of a mediastinal mass. A drill-biopsy of the mass did not yield conclusive proof of lymphadenoma, which was nevertheless probable on clinical grounds. Following treatment the itching was reduced and the skin, which was dry and ichthyotic, became more normal in texture. Some reduction in the mediastinal mass occurred (Fig. 5). The relief of symptoms lasted less than 3 months. This patient showed an interesting initial rise in the polymorphonuclear count at 4 days, prior to the onset of leukopenia. Lymphocytes and eosinophils were markedly reduced; both recovered to pre-treatment levels at 2 months. The effects in this case were about equivalent to those which would be expected with nitrogen mustard.

Case XI. Generalized lymphadenoma.—This patient, a biopsy-proved case, had previously been treated with local deep X-ray therapy and later on two occasions, with nitrogen mustard. On both these occasions improvement in well-being occurred and some reduction took place in the size of his enlarged liver, the improvement after the second course of nitrogen mustard being much less. On admission for treatment with 9500, the liver was enlarged and oedema of the legs was present. A transfusion preceded treatment, and this obscured the effect of the drug on the red cell count and haemoglobin. A short remission was obtained: the liver became smaller, the oedema disappeared and the patient felt better. The total effect obtained was comparable with that following nitrogen mustard with this difference, that nausea and vomiting had accompanied the previous treatments.

Multiple myeloma.

Case XII.—This section-proved case with positive Bence-Jones test showed no subjective or objective improvement following treatment. X-ray therapy given coincidentally to one of her lesions reduced the local pain and swelling.

Case XIII.—This patient, also a section-proved case, but with a negative Bence-Jones test, showed considerable symptomatic improvement in that the mobility of his shoulder was increased and pain was lessened. There was no radiographic evidence of restoration of the affected scapula. However, 5 months later the radiographs did not show any advance of the lesion.

Miscellaneous carcinomata.

Cases XIV, XV and XVI.—These cases represented various advanced stages of epithelial malignant growths. None of them responded to the drug. Case XV was later given a second course of treatment simultaneously with X-ray therapy, both agents being given as weekly treatments for growth-restraint. The drug was always injected before the radiation was given. The amount of growth-restraint so obtained was slight and no more than would have been expected from the radiation alone.

EFFECT ON NON-LEUKAEMIC BLOOD.

The effect on the non-leukaemic blood count is superficially similar to that which follows other cytotoxic agents, e.g. the nitrogen mustards or total body irradiation when these agents are used at dose levels in the clinical range. Urethane on the other hand, affects the normal blood count less.

The red cell count and haemoglobin percentage fall in most cases and recovery to pre-treatment levels may take several months. A leukopenia is generally seen, especially at higher dose levels. This is due to a fall in both granular and lymphocyte series.

The fall in the granular cells may be preceded by a transitory rise over 3 to 4 days, not seen unless blood counts are repeated soon after treatment. The minimum count occurs between 2 and 3 weeks following treatment; thereafter recovery begins (Fig. 6).

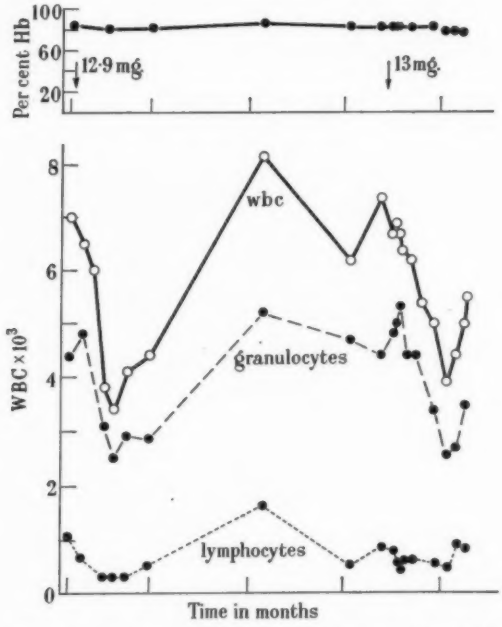

FIG. 6.—Case X: Lymphadenoma. Effect of 9500 on peripheral blood count in a non-leukaemic case.

No initial rise has been seen in the lymphocyte count, which reaches the lowest level 1 to 7 days after the end of treatment. Complete recovery is slow and would seem to take about 2 months. The response of other types of cells, large mononuclear and eosinophil granulocytes is irregular. Platelets were unaffected in all cases except one, in which a temporary diminution occurred.

A more exact comparison of the effects of 9500 and other cytotoxic agents would require a much larger series of cases.

DISCUSSION AND SUMMARY.

The value of a new chemotherapy agent against malignant disease depends on its superiority to radiation treatment or to those chemicals which have already

been evaluated clinically. It cannot be said on the evidence presented that trisethyleneimino-s-triazine is this superior agent.

In its fairly immediate effects on cases of leukaemia, of polycythaemia and of Hodgkin's disease, and in one case of myelomatosis, 9500 would seem about equal to the established methods of treatment. The long-term effects have not yet been studied.

The effects on the normal blood count resemble closely those obtained with the nitrogen mustards, or with irradiation when given in certain ways, in that the full effect on the white cell count is not seen until 2 to 3 weeks have elapsed. Individual variation in the response to the drug occurs although probably to no greater extent than obtains with other methods of treatment. For these two reasons we have waited for several weeks before instituting a second course of treatment in any patient.

An extended clinical trial of 9500 would, however, seem to be justified on several grounds: First because it may be found effective in diseases other than those quoted; even among the cases described there would seem to be a place for it in the treatment of polycythaemia. Secondly, it could be regarded as a pleasanter alternative to nitrogen mustard, since, unlike nitrogen mustard, 9500 does not produce gastric effects and there is no fear of local thrombosis. Thirdly, the drug is active if given by mouth; we have so far not tried its clinical effectiveness by this method, since animal experiments have demonstrated that by this route the haematological response was less predictable.

There is one disadvantage to an easily given drug of such potency. It is possible that it might be used without the constant supervision and check of blood counts which is necessary. As with all cytotoxic agents, its use should be contemplated only by those with facilities for adequate laboratory examinations.

The tissue culture work has been carried out by M. V. Thompson, the many blood counts by the Haematological Department of the Hospital, and the work on rat lymphosarcoma by Dr. H. Jackson. To all of these the authors acknowledge their indebtedness.

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REFERENCES.

Burchenal, J. H., Crossley, M. L., Stock, C., and Rhoads, C.—(1950) *Arch. Biochem.*, **26**, 321.

Idem, Johnston, S. F., Cremer, M. A., Webber, L. F., and Stock, C. C.—(1950) *Proc. Soc. exp. Biol., N.Y.*, **74**, 708.

Lewis, M. R., and Crossley, M. L.—(1950) *Arch. Biochem.*, **26**, 319.

Rose, F. L., Hendry, J. A., and Walpole, A. L.—(1950) *Nature*, **165**, 993.

COEXISTENCE AT ONE SITE OF TWO NEOPLASMS, ONE OF LOCAL ORIGIN AND ONE METASTATIC.

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THE occurrence of two neoplasms at one site, one being of local origin and the other a metastasis from a primary growth in another part of the body, is uncommon. Willis (1934) described nine such cases within his own experience, and accepted twenty-seven examples which had been reported by other authors. In these cases metastases from other tumours had developed in or invaded adenomatoid areas of the thyroid gland (12 cases), adrenal cortical adenomata (8 cases), ovarian cysts (6 cases), and uterine myomata (5 cases); there were also two examples of metastasis within a renal carcinoma, and one example each of a metastasis in a fibroma of the groin, in a cavernous angioma of the liver, and in a meningioma. It is noteworthy that involvement of "adenomata" or adenomatoid lesions of the thyroid and adrenal glands accounted for 20 of the 36 cases; in view of the doubt as to whether such tumour-like masses in endocrine glands are true neoplasms it is of interest that some of the other lesions in which metastases were found were also probably not truly neoplastic. In the literature since 1930 we have found only one further report (Mondor, Gauthier-Villars and Monsaingeon, 1938) of metastatic invasion of one neoplasm by another. In this case there was carcinomatous involvement of a large uterine "fibroma," associated with widespread carcinomatous infiltration of the myometrium and so-called Krukenberg tumours of the ovaries; it was claimed that these lesions were secondary to a primary carcinoma of the small intestine, which was thought to be unrelated to co-existent bilateral scirrhous mammary carcinomata.

The following 3 cases present several unusual features.

REPORT OF CASES.

Case 1.

B—, born in 1902, was in good health until May, 1948, when he developed symptoms of a progressive lesion of the spinal cord. His condition rapidly worsened, jaundice developed, and multiple, firm, subcutaneous nodules appeared. He died 3 months after the onset of the illness. The clinical diagnosis was carcinomatosis of unknown origin.

Necropsy (QEH 2942).

The body was emaciated, and weighed 57 kg. There was generalized icterus. Numerous, ill-defined, subcutaneous tumour nodules, each about 1 cm. in diameter, were present, particularly on the front of the trunk.

The upper air-ways, mouth and cervical organs were normal. The bronchi contained a little mucus. The right lung and the upper lobe of the left lung were congested. The lower lobe of the left lung was completely collapsed. The main bronchus of this lobe was occluded, 4 cm. below the tracheal bifurcation, by a firm, greyish-white tumour. The tumour extended outwards for 1·5 cm. into the surrounding lung tissue, and in collar-fashion for 4 to 5 cm. along the bronchus, its branches and the contiguous blood vessels. The cut surface of the tumour was finely patterned by white striae, which separated softer, yellow or grey areas. The lymph nodes in the hilus of the left lung were replaced by a large mass of partly necrotic tumour tissue which extended into the posterior mediastinum, compressing and invading the adventitia of the great vessels and of the oesophagus. The tumour had penetrated the pericardial sac, which contained about 60 ml. of straw-coloured serous fluid, and there was extensive invasion of the myocardium; the heart was otherwise normal. Numerous deposits of tumour, up to 3 cm. in diameter, were present in the left pleura, especially over the intercostal spaces, and the pleural sac contained approximately 300 ml. of blood-stained fluid. The right pleura was normal.

There was a tumour of the sigmoid colon at the level of the brim of the true pelvis. Approximately three-fourths of the growth, which measured $4 \times 3 \times 2{\cdot}5$ cm., projected in polypoid fashion from the outer surface of the bowel. Its cut surface resembled that of the bronchial tumour, and the general appearances suggested that it was a metastasis. The mucosa was fixed to the tumour and slightly elevated; superficial ulceration involved an area of $2{\cdot}5 \times 2$ cm. The lumen of the bowel was not obstructed, and the mesocolic lymph nodes appeared to be normal.

The peritoneal cavity contained a small amount of straw-coloured fluid; the serosa was sparsely studded with tumour deposits which measured up to 1 cm. in diameter. The liver was greatly enlarged and weighed 5320 g. The greater part of its substance was replaced by large, rounded, coalescent masses of bile-stained tumour tissue which had undergone extensive necrosis. The surviving liver tissue was deeply bile-stained and congested.

The lymph nodes around the coeliac axis and head of the pancreas, in the porta hepatis and in the lesser omentum were enlarged, and their substance was replaced by tumour tissue. The para-aortic and iliac lymph nodes were involved to a less degree. There were no relevant abnormalities of the other abdominal and pelvic viscera.

The body of the 11th thoracic vertebra was almost completely destroyed by tumour, and had collapsed, with consequent pressure upon the spinal cord. The spinal canal and its contents were otherwise normal. No tumour was found elsewhere in the skeleton. The cranial contents were normal.

Microscopic Examination.

The bronchial tumour (Fig. 1), and all the tumour deposits with the exception of that in the colon, consisted of rounded and spindle-shaped cells with a dense, hyperchromatic nucleus and little or no discernible cytoplasm; very occasionally small foci of squamous differentiation were seen, but otherwise the growth was completely undifferentiated. The cells had no particular arrangement, except for poorly developed palisading alongside the hyaline stromal trabeculae. Stroma was most abundant in the bronchial growth. There were extensive areas of

necrosis, especially in the hepatic deposits. The appearances were characteristic of an anaplastic bronchial carcinoma of the "oat-cell" type, and confirmed the macroscopic diagnosis of primary bronchial carcinoma.

The greater part of the colonic tumour was of the same anaplastic type as the tumour deposits elsewhere. The mucosa overlying the mass, however, showed an abrupt transition at its periphery from the normal structure and cytology to a polypoid, hyperchromatic columnar-cell adenocarcinoma, irregular gland-like extensions of which penetrated deeply into the tunica muscularis; the structure and cytology were those of a primary colonic adenocarcinoma. The stroma of the deeper parts of this growth was invaded by the anaplastic tumour, but no transition from one to the other was seen (Fig. 2). Only in one area had the undifferentiated tumour broken through to the lumen of the bowel; most of it lay external to the musculature.

In addition to the macroscopic metastases, rounded undifferentiated tumour cells were present within the lumen of many of the capillary blood vessels of the kidneys and myocardium, in the lymphatics and capillaries of the lung adjacent to the bronchial tumour, and in the splenic sinusoids.

Case 2.

G— (Hospital Registration Number C.57509), born in 1899, began to notice blood in his stools in 1945. One year later a carcinoma of the rectum was found and abdomino-perineal excision of the rectum performed. The patient remained in seemingly good health for more than 18 months, and then sustained a fracture of the pelvis in an accident. Nine weeks later an abscess developed deep in the inter-ischial region, necessitating incision; its contents were not examined. During the next fortnight a papilloma, about 0·8 cm. in diameter and situated at the medial end of the left inguinal skin fold, began to grow rapidly; it doubled its size within this period, after which it was excised. The papilloma had been present for at least 20 years before the first symptoms of rectal cancer occurred. There were no other skin lesions. The patient's condition deteriorated, metastases developed in the lymph nodes in each groin, and he died a few months later. There was no necropsy.

Microscopic Examination.

The primary rectal growth was a Grade 2 adenocarcinoma which had infiltrated through the bowel wall. The nearest lymph nodes were involved.

The skin tumour (Fig. 3) had the structure of a squamous papilloma; its connective-tissue core, however, was widely infiltrated by adenocarcinoma (Fig.

EXPLANATION OF PLATES.

FIG. 1.—Case 1. Anaplastic bronchial carcinoma. H. and E. × 188.

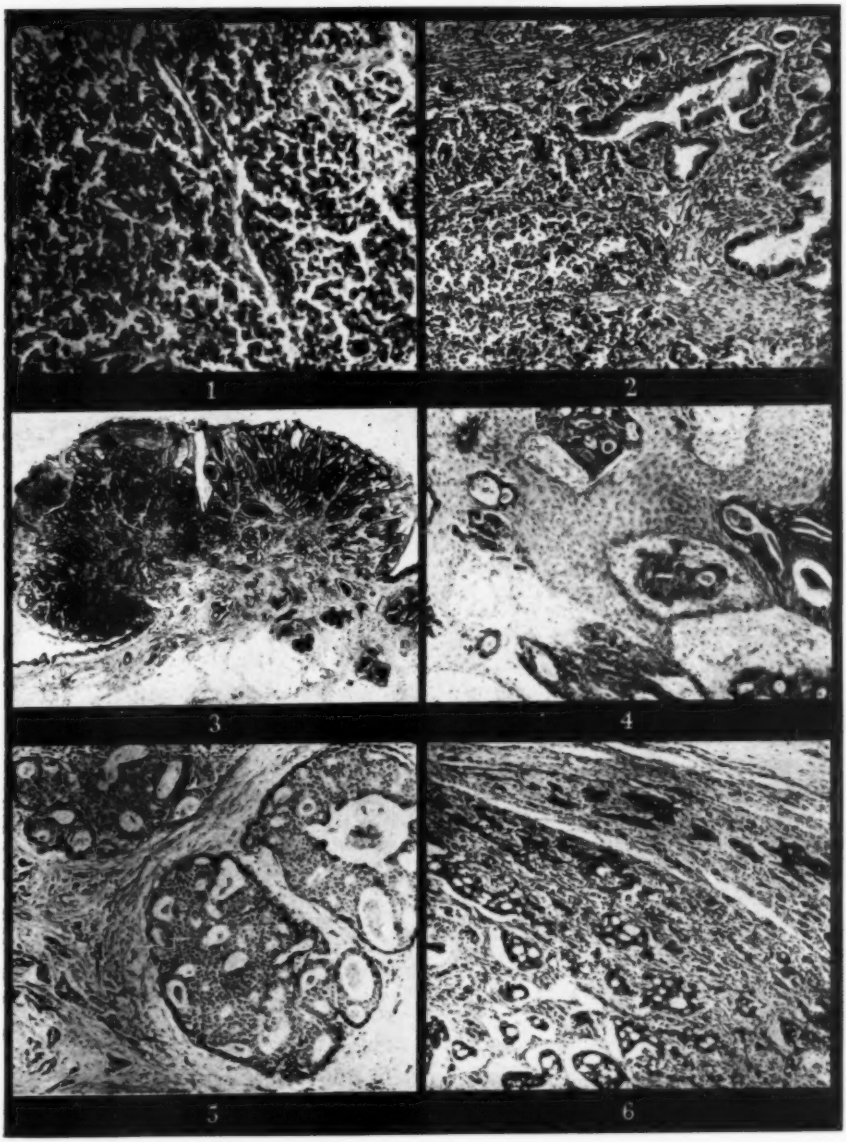
FIG. 2.—Case 1. Infiltration of primary adenocarcinoma of colon by metastatic anaplastic carcinoma. H. and E. × 90.

FIG. 3.—Case 2. Squamous papilloma of groin, with infiltration of its core by metastatic adenocarcinoma. H. and E. × 3·3.

FIG. 4.—Case 2. Adenocarcinomatous infiltration of stroma of squamous papilloma. H. and E. × 93.

FIG. 5.—Case 3. Cribriform and undifferentiated scirrhous carcinoma of breast. H. and E. × 93.

FIG. 6.—Case 3. Metastatic carcinoma in sclerotic ovarian cortex, with infiltration of fibrotic "dermis" of cystic teratoma. H. and E. × 93.



Jackson and Symmers.

4), the structure of which was identical with that of the rectal tumour. There was no evidence of malignant change in the squamous epithelium.

Case 3.

L— (Hospital Registration Number G.115205), born in 1896, noticed a tumour in her right breast in 1940. Radical mastectomy was performed 2 years later, and at the same time a small tumour was excised from the left breast. The patient then remained apparently well until 1949, when she began to be dyspnoeic ; examination showed a right-sided pleural effusion. Clear, amber-coloured fluid was aspirated 5 times, at intervals of a few weeks, the quantities withdrawn ranging from 550 to 1200 ml. The fluid contained between 4 and 5 per cent of protein ; a few cells were present, mostly lymphocytes, with a very small proportion of neutrophile polymorphonuclear leucocytes and normal serosal cells. Aerobic and anaerobic cultivation on ordinary media yielded no growth, and cultures for *Mycobacterium tuberculosis* were also sterile. Radiography showed no evidence of neoplasm in the chest, but the effusion persistently obscured the greater part of the right lung field ; there was never any demonstrable effusion in the left pleural sac.

As there was no evidence of recurrence or metastasis of the carcinoma, and as there was no demonstrable infective cause of the recurrent pleural effusion, the discovery of an adnexal tumour on pelvic examination suggested the possibility that the condition was an atypical variant of the Meigs-Cullingworth syndrome. At laparotomy, 6 months after the onset of dyspnoea, a cystic tumour of the left ovary was found and removed ; there was no other abnormality in the pelvis or abdomen. Aspiration of the pleural effusion on the day before laparotomy yielded 1050 ml. of fluid, which, for the first time, was haemorrhagic ; laboratory examinations again showed no evidence of neoplasia or infection causing the effusion.

Convalescence from the laparotomy was uneventful, but after the patient returned home the unilateral pleural effusion recurred, necessitating repeated thoracenteses ; the fluid remained heavily blood-stained. Apart from the pleural effusion there were no clinical manifestations to suggest the presence of a neoplastic process. The patient died 3 months after the oophorectomy ; as there was no necropsy the existence of metastases could not be confirmed.

Pathological Findings.

The tumour in the right breast was a predominantly intraduct adenocarcinoma, with widespread scirrhous infiltration of the breast stroma (Fig. 5). Metastases in the axillary lymph nodes reproduced both the cribriform and the scirrhous structure. The tumour of the left breast was a simple intracanalicular fibro-adenoma.

Macroscopically, the ovarian tumour was a unilocular cystic teratoma, 11 cm. in diameter, filled with sebum-like material and hair. The cyst had a smooth lining ; its wall was 1 mm. or less in thickness, except near the pedicle, where there was an irregular, bone-hard eminence, approximately 2 cm. in diameter, projecting into the cavity. Adjacent to the pedicle the remnant of the ovary was identifiable on the external surface of the tumour as a flattened, ovoid area of firm, greyish-white tissue, which measured 3·5 × 2·5 cm. superficially, and up

to 0·4 cm. in thickness. Microscopically the cyst was lined by keratinizing epidermis, external to which was a narrow zone of fibro-fatty tissue. Hair-follicles and large sebaceous glands were numerous in the skin covering the hard projection, which consisted of bone, bronchial structures and neuroglia. The ovarian remnant was sclerotic, and widely infiltrated by carcinoma which reproduced the cribriform adenocarcinomatous structure and the undifferentiated growth seen in the tumour of the right breast (Fig. 6). The carcinoma extended widely into the adjacent " dermis " of the teratoma, and into the tissues of the intracystic projection.

DISCUSSION.

Case 1.

Three possibilities require to be considered in interpreting the findings in this case: (1) A single primary carcinoma, arising in the bronchus, retaining its anaplastic characteristics in all the metastases except that in the colon, and in the latter situation differentiating to form an adenocarcinoma which in structure exactly mimicked a characteristic colonic cancer; (2) a single primary carcinoma, arising in the colon, undergoing anaplasia, and in its metastases perfectly reproducing the typical anatomical and histological appearances of a primary bronchial carcinoma; (3) two primary carcinomata, one originating in the bronchus and giving rise to the metastases, and the other developing in the colon at the site where a metastases from the bronchial tumour was also found. The third hypothesis seems to us to be the only one tenable.

If the coexistence of two primary neoplasms is accepted, the significance of the presence of a metastatic deposit from the primary bronchial carcinoma at the site of the primary colonic carcinoma requires to be considered. This association may have been fortuitous, or the presence of the colonic neoplasm may have predisposed to the establishment there of the metastasis, or the development of the latter may have induced cancerous change in the adjacent mucosa; it seems to be impossible to say which suggestion is correct. The induction, by some effect of the existing neoplasm, of sarcomatous change in the stroma of carcinomata has at times been postulated in explanation of the genesis of so-called carcinosarcomata: in view of the considerable doubt as to the origin of the sarcoma-like component of these tumours, such a hypothesis has little practical value. The induction of one carcinoma by another was postulated by Dubois-Ferrière (1939), who suggested that hepatic metastases in his case of islet-cell pancreatic carcinoma had induced local development of bile-duct carcinomata. He was not able to exclude the possibility that the adenocarcinomatous areas in the liver were metastases with an unusually well-developed tubular structure, such as is sometimes seen in islet-cell neoplasms.

Seecof (1924) reported a case which was in some respects similar to ours. A large tumour was present in the right lung, involving the main bronchi and destroying most of the middle lobe and the adjacent parts of the other lobes. This tumour and the metastases in the bronchial lymph nodes, adrenal glands, omentum, pancreas and pelvis, consisted of large, rounded and polygonal cells; the photomicrographs illustrating Seecof's (1924) paper show that the cytology was quite unlike that of the usual types of bronchial neoplasm. There was also a large, well-differentiated rectal adenocarcinoma, which had invaded the neighbouring tissues; metastases in the left lung were histologically identical with

this rectal tumour. Seecof (1924) considered that the tumour in the rectum and that in the right lung were independent primary neoplasms, the former giving rise to the adenocarcinomatous metastases in the left lung, and the latter to the undifferentiated metastases, including the large mass in the pelvis which had invaded the primary rectal carcinoma. Willis (1934) comments that Seecof's (1924) diagnosis is open to serious doubts. In our case each carcinoma was of a distinctive type, well recognized as characteristic for the site concerned, and the evidence in support of the presence of two primary carcinomata seems to be considerably stronger.

Our other cases require little comment, and they have been recorded here only because of the rarity of the phenomenon which they represent.

Case 2.

Growth of the metastatic carcinoma in its core presumably accounts for the rapid enlargement of the papilloma, which doubled its size in the 2 weeks preceding its excision, after having been present unchanged for many years. The metastasis in the papilloma was microscopically identical with the rectal carcinoma ; the structure was not that of an adenocarcinoma arising in the skin. There was no sign of cancerous change in the squamous epithelium.

Case 3.

The fact that the complex microscopic appearance of the ovarian carcinoma was identical with that of the primary mammary carcinoma, radically resected 7 years earlier, makes it reasonably certain that the carcinomatous growth in the ovary originated as a metastasis from the breast tumour, although a local origin in the teratoma or possibly in the remnant of ovarian tissue should not, perhaps, be absolutely excluded. Accepting the metastatic nature of the carcinoma in the teratoma, this case is also of interest in respect of the latency of the cancer for 7 years after radical mastectomy, and because the coexistence of pleural effusion and the adnexal tumour suggested the possibility of an atypical variant of the Meigs-Cullingworth syndrome in explanation of these findings. This syndrome was finally excluded by the course of the illness after removal of the ovarian tumour, and by the haemorrhagic nature of the effusion in its later stages.

It is of some interest in relation to this case that Küster (1911) reported a case in which metastases from a primary gastric carcinoma developed in both ovaries, involving a typical " dermoid cyst " which was present in one of them.

SUMMARY.

Three cases are reported in which there was coexistence in one site of two neoplasms, one of which was of local origin and the other metastatic.

In the first case an anaplastic bronchial carcinoma gave rise to multiple metastases, one of which was in the wall of the colon at the site of an adenocarcinoma arising in the intestinal mucosa. It is impossible to say whether this coexistence was fortuitous ; it is possible that development of the intestinal carcinoma was induced by the metastatic carcinoma present in the same site.

In the second case a metastasis from a primary carcinoma of the rectum

developed in a squamous papilloma which had been present in one groin for at least 20 years.

In the third case a metastasis from a mammary carcinoma developed in the wall of a typical unilocular cystic teratoma of one ovary.

REFERENCES.

DUBOIS-FERRIÈRE, H.—(1939) *Helv. med. Acta*, **6**, 458
KÜSTER, H.—(1911) *Z. Geburtsh. Gynäk.*, **68**, 364.
MONDOR, H., GAUTHIER-VILLARS, P., AND MONSAINGEON, A.—(1938) *Ann. Anat. path. méd.-chir.*, **15**, 932.
SEECOF, D. P.—(1924) *J. Cancer Res.*, **8**, 213.
WILLIS, R. A.—(1934) 'The Spread of Tumours in the Human Body.' London (Churchill), pp. 413, 414.

INVESTIGATION OF THE EFFECT OF X-RADIATION ON THE LOCALIZATION OF RADIOACTIVE PHOSPHORUS IN BREAST TUMOURS.

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THE distribution of radioactive phosphorus can be investigated *in vivo* using a thin-walled G.M. counter tube. The maximum range for beta particles of P_{32} in soft tissue is 8 mm., but the half-value thickness is only of the order of 0·6 to 0·8 mm. Consequently, measurements on the skin can only indicate the amount of phosphorus in the superficial layers of the body (effectively some 3 mm. deep). However, under favourable conditions, such measurements can be used to demonstrate the increased phosphorus up-take by malignant tumours. Low-Beer, Glenn-Bell, McCorkle and Stone (1946) have used this method with some success, as a diagnostic procedure, but its value is reduced by the low penetration of the beta rays and by the increased up-take also found in inflammatory conditions. The present series of experiments (started in November, 1948) was undertaken to investigate possible changes in the phosphorus content of malignant tumours during and after a course of X-ray treatment, and to attempt to correlate such changes with the reaction of the tumour to the irradiation.

MATERIAL AND METHOD.

Only cases of mammary carcinoma with the tumour attached to the skin were selected; the patients were in good general health.

On each occasion a tracer dose of about 150 μc. P_{32} was given by intravenous injection in 5 c.c. phosphate buffer at pH 7·35. It has been found advisable to check, by counter measurements, that the radioactive material has actually left the site of injection, as, even after taking more than ordinary care, some unsatisfactory injections have been detected. This is particularly important, of course, when giving the isotope therapeutically, as dose calculations assume that the whole of the material enters the blood stream. No side effects of the injection were observed. Assuming uniform distribution, a body weight of 60 kg. and a biological half life of 8 days, the dose delivered was about 0·1 r in the first day and about 1·1 r over a period of some 4 weeks. The tumour may have received 4 to 8 times the dose calculated above, a total which is in excess of the generally agreed "tolerance" level. This was, however, of no consequence as the site was to receive an X-ray dose of 3000 to 4000 r.

A number of sites over the tumour, on the diseased breast, and at corresponding points on the normal breast, were marked with gentian violet, and measurements of the activities were made with a counter tube applied directly

to the skin. A GM4 counter (General Electric Co., Ltd., England) was used, with conventional quenching and scaling circuits, the 0·001 inch thick duralumin window being covered with a sheet of cellophane to give some protection, and to avoid possible contamination of the window by the skin.

Some difficulty was experienced because of the rise of temperature of the counter in contact with the skin ; this led to a slight fall in count rate due to a shift of the counter voltage characteristic. In order to ensure that the operating point did not leave the plateau of the characteristic curve, the initial setting was some 150 volts above the critical voltage. The counter was checked against a standard beta ray source (uranium oxide with filter to remove alpha rays) before, and at intervals during the skin measurements, and the operating voltage adjusted slightly to maintain the same count rate. The background rate was checked before and after the series. In each case the complete set of measurements was repeated and a mean value for each site was used. It was not feasible to prolong the counting time at any site above about 3 minutes. As the count rates with this tracer dose were rather low, the measurements were usually limited to 1000 counts (3 per cent standard deviation) or, after several days and over normal tissue, to only 100 counts (10 per cent standard deviation). Consequently, the ratios of count rates over tumour and normal skin were not obtained very accurately, but the results sufficed to demonstrate large changes.

Series of measurements were made before, during, and 4 to 6 weeks after the treatment, at which time the X-ray erythema had subsided but the skin of the irradiated breast remained highly pigmented. Observations were made on alternate days during 2 to 3 weeks.

After intravenous injection, the radioactive phosphorus could be demonstrated immediately (5 to 8 seconds) in the tissues of the breast, but the concentrations continued to increase in both healthy and diseased breasts for 10 to 15 minutes. Considerably lower count rates were observed when the next series of measurements were made after an interval of 2 days, a change probably due to the excretion of P_{32}. Thereafter the fall was found to follow a roughly exponential form, corresponding to a " biological half-life " of between 7 and 9 days. Typical cases are illustrated in Fig. 1. The excretion of P_{32} was not followed up with patients, but in preliminary experiments with rats it was found that, after intra-cardiac injection, a large percentage of P_{32} was excreted in the urine in the first 48 hours. After that time, no P_{32} could be demonstrated in the urine, but a small quantity continued to be found in the faeces over a period of at least 30 days.

The X-ray treatment was the same in Cases 1 to 4. Tumour dose of 3400 r (200 Kv., 1·0 mm., Cu. H.V.L.) was given (fractioned as 260 r on alternate days) using a glancing field technique. With Case 5 the whole chest wall received low voltage irradiation (140 Kv., 0·25 mm., Cu. H.V.L.) to a total of 2500 r in 5 weeks.

Case records.

(1) *Mrs. L. C—*, aged 57. Tumour in the left breast ; histologically solid trabecular, polygonal-celled carcinoma. The tumour was at least 15·0 cm. in diameter, hardest in the upper and outer quadrant. The skin was extensively involved. The mass was attached to muscle but not to the chest wall.

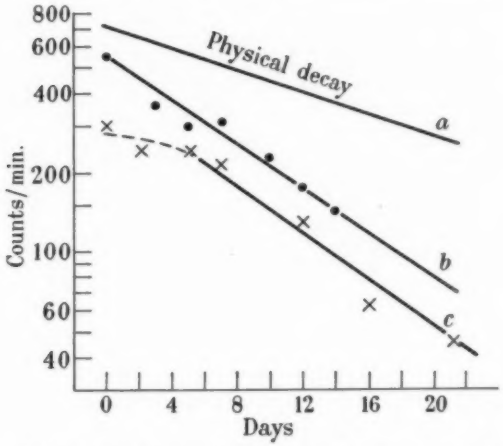

FIG. 1.—Fall of count rate over tumours in two typical cases as compared with physical decay.

(*a*) Physical decay.
(*b*) and (*c*) Count rates in two patients when not receiving X-ray treatment.

The actual count rates before and during the X-ray treatment are given in Table I. Apart from the breast measurements, readings taken over the forehead

TABLE I.—*Average Counts/Minute Above Background.*

Site.	Before and during X-ray treatment.								
Date	18.ii.	21.ii.	23.ii.	25.ii.	X-ray treatment started.	28.ii.	2.iii.	4.iii.	7.iii.
Tumour	801	260	240	197		118	86	61	42
Tumour edge	563	124	108	94		66	50	45	36
Diseased breast	635	117	89	66		53	51	41	—
Normal breast	359	87	63	77		63	41	41	28
Normal breast	171	87	65	50		41	48	42	33
Axilla, tumour side	500	122	149	102		64	74	47	32
Axilla, normal side	430	159	149	98		80	76	36	—
Forehead	444	324	275	200		162	147	118	109

and over both axillae are also included. It is of interest to note that the readings over the forehead (indicating the P_{32} content of the frontal bone) increased to the level found over the tumour and then decreased more slowly than the latter. Values of the ratio of count rates over the diseased breast to those over the healthy breast are presented in Fig. 2*a* and 2*b*. Before treatment the ratio of the count rates remained constant, but a break in the curves was observed 3 days after the irradiations started. The measurements in Fig. 2*b* were made 6 weeks after the end of the X-ray treatment, when the whole surface of the irradiated breast had a higher P_{32} content than the healthy breast, the ratio between the two being similar to that before treatment.

The patient's condition deteriorated, and 7 months after treatment she died with extensive ulcerative carcinoma.

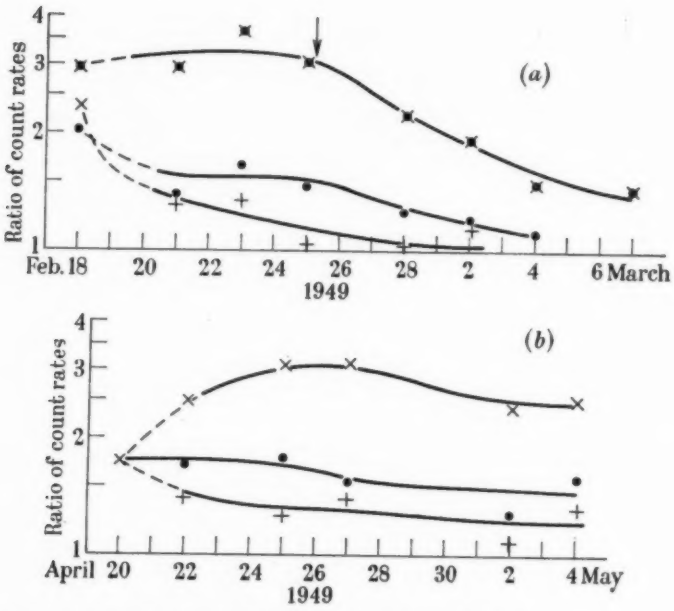

FIG. 2.—Ratio of count rate over diseased to that over normal breast.
Mrs. L. C— :

—— × —— × —— × —— Ratios over tumour.
—— ● —— ● —— ● —— Ratios over tumour periphery.
—— + —— + —— + —— Ratios over apparently normal tissue on diseased breast.

(*a*) Before and during X-ray treatment. Arrow marks first treatment.
(*b*) Six weeks after X-ray treatment.
The curve is shown dotted between the first two measurements after the injection of P_{32}, during which time the ratios were not steady.

(2) *Mrs. A. W—*, aged 60. Polygonal-celled carcinoma in the right breast. The tumour measured 11 × 9 × 6 cm. No palpable axillary glands.

In this, our first exploratory case, X-ray treatment started on the day following the injection of a tracer dose of P_{32}. The results are given in Fig. 3*a*. A sudden drop in the ratio occurred after the third irradiation. Four weeks after the end of the treatment a second tracer dose was given, and the results obtained are shown in Fig. 3*b*. For the first three readings the skin was still very red. By the time the erythema had subsided the count rates were rather low, but they appeared to be higher than normal over the whole of the diseased breast, without any localized area of high activity.

Two months after the irradiation total mastectomy was carried out. The patient is in good general condition with no evidence of recurrence $2\frac{1}{2}$ years after the treatment.

(3) *Mrs. F. N—*, aged 65. Patient had a large hard right breast, with peau d'orange, containing a tumour, with ulceration and infiltration of the skin. There was pleural effusion on the left side.

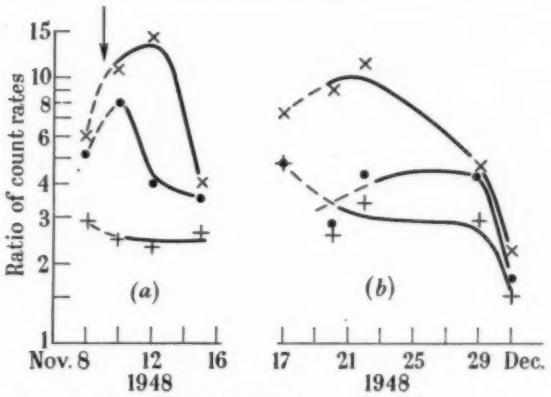

FIG. 3.—Ratio of count rate over diseased to that over normal breast.
Mrs. A. W— :

—— × —— × —— × —— Ratios over tumour.
—— ● —— ● —— ● —— Ratios over tumour periphery.
—— + —— + —— + —— Ratios over apparently normal tissue on diseased breast.

(*a*) During X-ray treatment. Arrow marks first treatment.
(*b*) Four weeks after X-ray treatment. Erythema still present.
The curve is shown dotted between the first two measurements after the injection of P_{32}, during which time the ratios were not steady.

The measurements with this patient are of particular interest as she received a course of stilboestrol before starting X-ray treatment. As shown in Fig. 4*a* the ratio of count rates over the two breasts remained constant (or even tended to rise) during the stilboestrol treatment, which produced little clinical improvement. Five weeks after the stilboestrol treatment was finished, X-ray treatment started. After two X-ray doses a sudden fall in the ratio was again observed (Fig. 4*b*), but a third series of measurements, 4 weeks after the completion of the X-ray treatment, indicated ratios as high as those obtained before treatment (Fig. 4*c*).

The patient did not improve considerably. One year after treatment she developed neurological symptoms and died.

(4) *Mrs. H—*, aged 72. Advanced carcinoma of the right breast, a diffuse thickening occupying most of the breast.

Fig. 5 shows the measurements over the diseased breast relative to those over the normal breast. The ratios, fairly constant before treatment, dropped suddenly after the second irradiation and continued to fall towards unity. Because of the age of this patient the injection of P_{32} was not repeated after treatment.

(5) *Miss E. R—*, aged 61. Stage III tumour of left breast.

Although in this case the *whole* chest wall was irradiated, the ratio of count rates over the diseased and normal breasts showed a sudden drop after the second irradiation (Fig. 6), indicating a diminution of P_{32} uptake in the affected as compared with the normal breast. Measurements were also made over an isolated skin nodule separated from the primary growth by 6 cm. of apparently normal skin. The initial count rate over this nodule was only slightly lower than that over the primary growth, and the exactly similar changes were observed for these two sites.

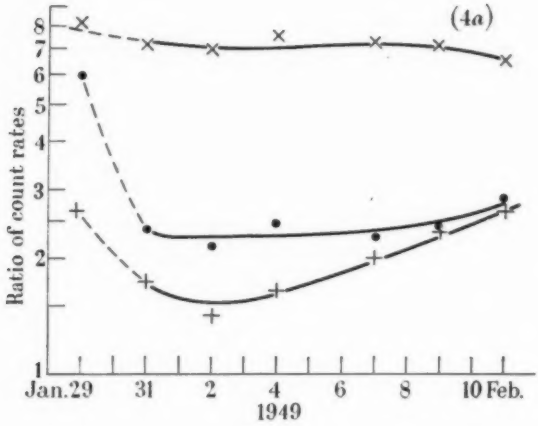

FIG. 4a.

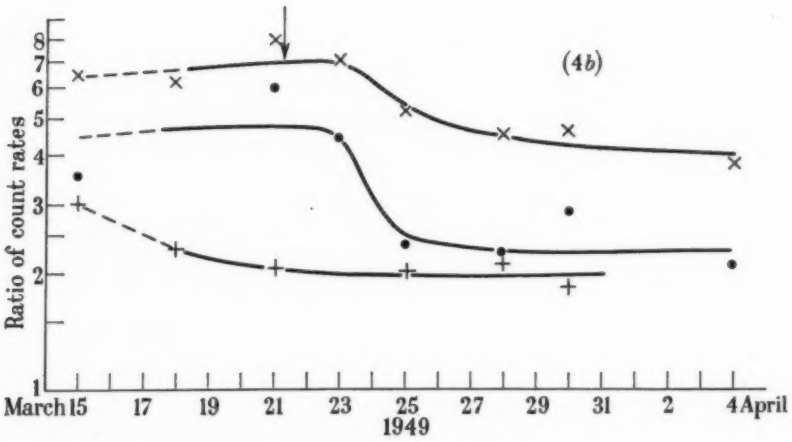

FIG. 4b.

FIG. 4.—Ratio of count rate over diseased to that over normal breast.
Mrs. F. N—:

—— × —— × —— × —— Ratios over tumour.
—— ● —— ● —— ● —— Ratios over tumour periphery.
—— + —— + —— + —— Ratios over apparently normal tissue on diseased breast.

(*a*) During stilboestrol treatment.
(*b*) Before and during X-ray treatment. Arrow marks first X-ray treatment.
(*c*) Four weeks after X-ray treatment.

The curve is shown dotted between the first two measurements after the injection of P_{32}, during which time the ratios were not steady.

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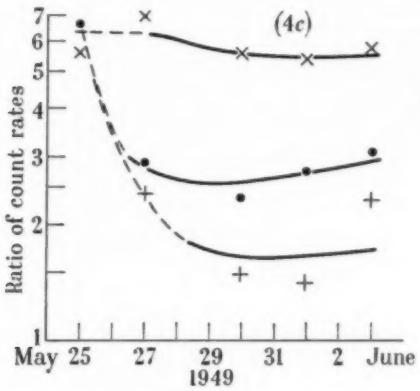

FIG. 4c.

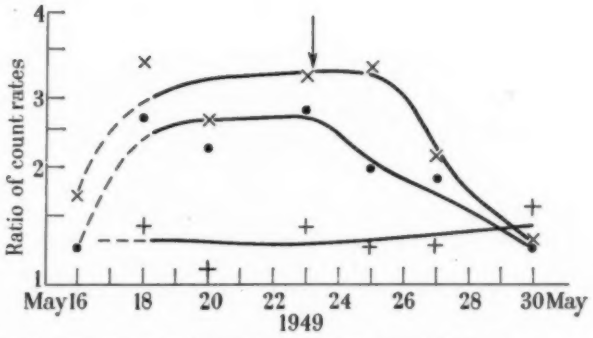

FIG. 5.—Ratio of count rate over diseased to that over normal breast.
Mrs. H— :

—— × —— × —— × —— Ratios over tumour.
—— ● —— ● —— ● —— Ratios over tumour periphery.
—— + —— + —— + —— Ratios over apparently normal tissue on diseased breast.

Before and during X-ray treatment. Arrow marks first treatment.
The curve is shown dotted between the first two measurements after the injection of P_{32}, during which time the ratios were not steady.

DISCUSSION.

Although the number of cases is very small, the results appear to be of sufficient interest to justify publication, particularly as the present series of experiments has had to be discontinued because the team has dispersed. At a later date the authors hope to continue the investigation, although working at different institutions.

The count rate due to P_{32} was found to be very much higher (between 3 and 8 times) over malignant mammary tumours attached to the skin, than over the corresponding parts of the healthy breast. A higher count rate was also recorded

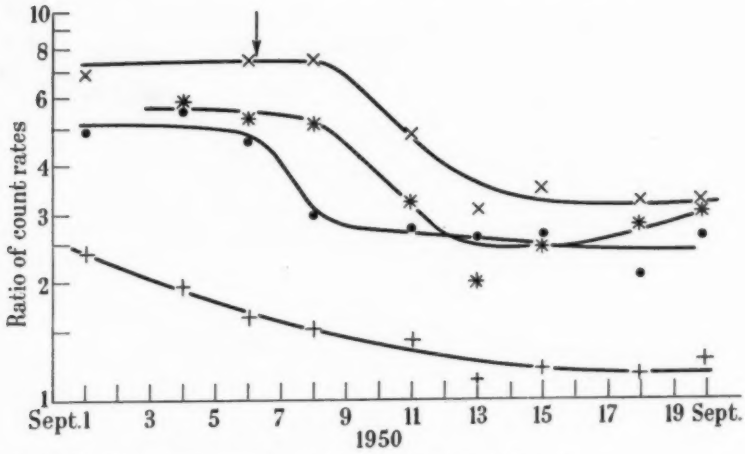

FIG. 6.—Ratio of count rate over diseased to that over normal breast.
Miss E. R— :

—— × —— × —— × —— Ratios over tumour.
—— ● —— ● —— ● —— Ratios over tumour periphery.
—— + —— + —— + —— Ratios over apparently normal tissue on diseased breast.
—— * —— * —— * —— Ratios over isolated skin nodule.

Before and during X-ray treatment. Arrow marks first treatment.

over parts of the diseased breast remote from the palpable tumour, but it is not possible to decide whether this is due to a greater tissue vascularity or to some microscopic or sub-microscopic spread of the tumour.

The exponential rate of disappearance of P_{32} was found to be approximately the same for diseased and normal breasts, but in all cases receiving X-ray treatment, a sudden fall in count rate over the tumour and over that breast was observed after the second or third irradiation.

It appeared that this sudden fall occurred first over the peripheral parts of the tumour, a drop over the central part following after one or two further irradiations.

In experiments with rats, X-radiation produced a marked inhibition of P_{32} uptake in the thyomonucleic acid fraction in tumours and other tissues with high mitotic activity (Hevesy, 1945 ; Holmes, 1947). Although a different technique was used, these findings may account for the results reported above. It is not possible to decide, from the present experiments, whether the changes observed during the course of X-ray treatment of patients are due to change in metabolism of the tumour cells or to vascular damage. However, it may be possible to decide this by suitably controlled experiments.

Count rates, much higher than normal, were observed over areas of X-ray erythema, and it is therefore important to postpone measurements after treatment until this reaction completely clears. The ratios of count rates over the two breasts were then found to be similar to those obtaining before X-ray treatment, but, as the primary growths were still present in all these cases, it is not

possible to say what the ratio would be after a treatment when the primary tumour has disappeared.

SUMMARY.

Tracer doses of about 150μc. P_{32} were given intravenously to five patients with malignant breast tumours attached to the skin, and measurements made with a beta counter applied directly to the skin. Series of measurements were made before, during, and after X-ray treatment, and, in one case, during a course of stilboestrol treatment. The count rate recorded was much higher over the tumour, and to a lesser extent over the whole of that breast, than over corresponding sites on the normal breast. The exponential rate of disappearance of P_{32} was found to be approximately the same for both breasts, but after the second or third X-ray treatment a sudden fall was observed in the count rate over the tumour and over that breast relative to that over the normal breast. This result was obtained in all cases, including one in which both breasts received the same low voltage (140 Kv.) irradiation. No such change occurred during stilboestrol treatment in a case which did not respond clinically. After erythema had subsided the ratios of measurements over the two breasts were found to be similar to those before treatment, but the primary growth was still present in all these cases.

The authors wish to thank Mr. Sykes, the chief pharmacist at the hospital, for preparing the material for intravenous injections, and Mr. K. Goodall who assisted with the experimental work. The work was largely financed by funds from the British Empire Cancer Campaign.

REFERENCES.

HEVESY, G.—(1945) *Rev. mod. Phys.*, **17**, 102.
HOLMES, B. E.—(1947) *Brit. J. Radiol.*, **20**, 450.
LOW-BEER, B. V. A., GLENN-BELL, H., MCCORKLE, H. M., AND STONE, R. S.—(1946) *Radiology*, **47**, 422.

THE INVASION AND CELL REPLACEMENT OF ONE PIGMENTED NEOPLASTIC GROWTH BY A SECOND, AND MORE MALIGNANT TYPE IN EXPERIMENTAL FISHES.

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THE invasion and replacement of one kind of tumour by another has rarely been reported in biological studies of neoplastic diseases. Such a phenomenon, involving the abnormal growth of two kinds of pigmented cells, was recently found in spontaneous tumours experimentally produced by genetic methods in platyfish-swordtail hybrids.

The tumorous hybrids were produced by mating a male spot-sided and ruby-throated platyfish (*Platypoecilus maculatus*) to an albino swordtail (*Xiphophorus hellerii*). The spot-sided pattern in the parent platyfish is made up of scattered macromelanophores, which are large black pigment cells containing melanin granules. The ruby-throated pattern is composed of xantho-erythrophores, which are red pigment cells containing erythropterin in granular form and lutein and zeaxanthin in solution. Gordon (1950) and Gordon and Nigrelli (1950) indicated that these colour patterns are inherited and referable to two platyfish sex-linked genes : *Sp* for the black and *Rt* for the red pigment cells. The genetic analysis revealed that the pigmented tumours were produced in platyfish-swordtail hybrids containing the linked genes, *Sp* and *Rt* from the platyfish and a number of *Sp* and *Rt* gene modifiers from the swordtail.

Some of these pigmented tumours were diagnosed as erythromelanomas in an earlier report by Gordon (1950) and Nigrelli, Jakowska and Gordon (1950). More extensive study of additional specimens revealed that there were two kinds of pigmented tumours involved, first, a red tumour, a xantho-erythrophoroma of the head and, second, a black tumour, a melanoma of the body. In some of the hybrids, cells of the melanoma reached, penetrated and replaced the tissues of the red pigmented tumour. When joined, the two pigmented tumours produced an erythromelanoma. The purpose of this paper is to present a more detailed histological and cytological analysis of these pigmented tumours and to suggest a genetic interpretation of the developmental aspects of the problem.

MATERIAL AND METHODS.

The genetic history of the strain of hybrid fishes that spontaneously developed red tumours was reported by Gordon (1950), a summary of which is indicated in Fig. 1.

Eight platyfish-swordtail hybrids with red or red-black tumours were found in a single brood in the first generation. Of these, 3 are described in this histological analysis. In the second generation 11 hybrids had unmistakable signs

of red tumours, of which 3 are here described. In a backcross of one of the tumorous first generation hybrids to a normal swordtail, 5 additional individuals had tumours. When one of the second generation males was bred back to a normal swordtail, 3 more hybrids developed the same kind of pigment cell tumours, one of which is here described.

A total of 27 fishes with red or red-black tumours was obtained by genetic methods. It was not feasible to section all the animals with the red abnormal

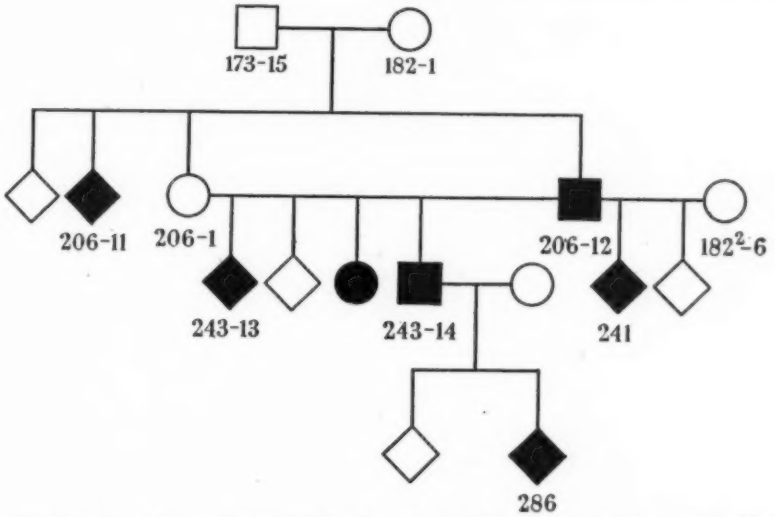

FIG. 1.—*First line :* Parental types : 173–15 represents the normal, red-throated, spot-sided male platyfish ; 182–1 represents the normal, albino female swordtail (Fig. 34). *Second line :* First generation platyfish-swordtail hybrids : 206–11 developed a xantho-erythrophoroma ; 206–12 developed an erythromelanoma (Fig. 36, 37) ; 206–1 is a normal recessive hybrid (Fig. 38). *Third line :* Second generation hybrids (pedigree No. 243) to the left and backcross generation hybrids (pedigree No. 241) to the right. (Hybrids of pedigree No. 241 are illustrated by Fig. 39). *Fourth line :* Backcross generation of pedigree No. 286, produced by the mating of a second generation hybrid, 243–14, to an albino swordtail.

□ Male. ○ Female. ◇ Indeterminate. ◆ Tumorous.

growths, but 7 representative specimens were chosen, fixed, sectioned and studied. The first generation hybrids so studied had the following numbers : 206–11, 206–12 and 206–14. The second generation hybrids were 243–11, 243–12 and 243–13. The backcross offspring of a second generation hybrid mated to a swordtail was 286. Some of these fishes are illustrated in Fig. 34 to 39.

As Gordon (1950) indicated, not all hybrids carrying *RtSp* developed typical erythromelanomas or xantho-erythrophoromas. Many of these animals were classified as being in a state of melanosis or simple melanoma. These melanotic animals presented features previously described by Gordon and Smith (1938) and Levine (1948) and are not further described here.

Tumorous fish were first biopsied and later autopsied for microscopical examination. The biopsied material was studied in the fresh state with ordinary light

and by phase contrast. In order to obtain better cellular details in the non-fixed material and to identify the pigments, pieces of skin and isolated scales were treated with acetone to extract the xanthophyll and with dilute ammonia to remove the pterins from the pigmented cells. This was the initial procedure recommended by Goodrich, Hill and Arrick (1941) for the extraction and chemical identification of these pigments from the skin of fishes.

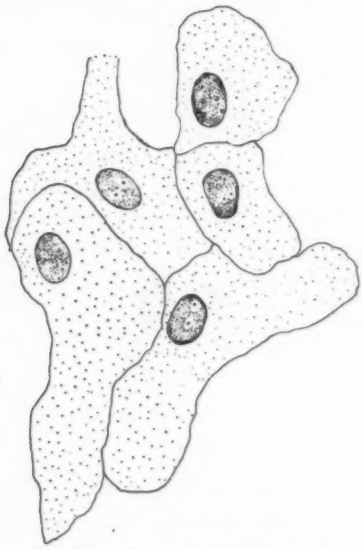

FIG. 5.—Normal xantho-erythrophores from isolated scales of a domesticated red swordtail. Haematoxylin eosin. × 1000.

Tissues were fixed in formalin or in Bouin's solution. They were decalcified, embedded in paraffin and sectioned at 5 μ. Sections were stained with Heidenhain's iron-haematoxylin, Harris's haematoxylin and Delafield's haematoxylin, each with and without eosin. Mallory's, Masson's, and Giemsa's staining methods were also used. Material used for Feulgen smears was fixed in 3:1 alcohol-acetic acid solution. Parts of the tumours were treated in bulk for other Feulgen preparations, embedded in paraffin and sectioned.

HISTOLOGICAL AND CYTOLOGICAL OBSERVATIONS.

The *RtSp* platyfish-swordtail hybrids of the present series show several possible courses of development. Some may develop melanomas, others may develop xantho-erythrophoromas in the opercular area of the head. The red tumours may be invaded by melanotic cells and eventually replaced in part by melanoma cells while others may continue to grow as xantho-erythrophoromas.

The normal xantho-erythrophore in the living, domesticated red swordtail is a highly dendritic cell with coarse red granules and with yellow pigment diffused

in the cytoplasm (Fig. 2). When xantho-erythrophores are involved in the tumour formation as, for example, in Hybrid 286, they are pleomorphic ; those that are isolated from the main tumour mass round up and disintegrate rapidly (Fig. 3, 4). In fixed and stained isolated scales from normal skin, the xantho-erythrophores show no dendrites. Their nuclei are spherical or oval ; they stain weakly and contain fine chromatin granules with two or more darker staining bodies (Fig. 5).

Histologically, the simple xantho-erythrophoromas, as represented in Hybrids 243–12 and 243–13 (Gordon, 1950), occupy the corial region of the skin of the

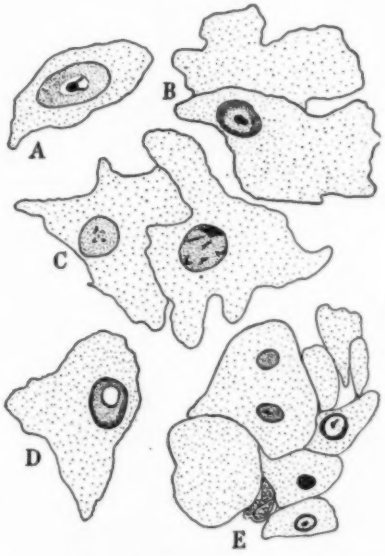

FIG. 12.—Cells from early stage of xantho-erythrophoroma (243–13). A–D, Cells showing different nuclear patterns ; E, cells showing binucleated condition and pycnosis. Note macrophage. Masson's. × 1000.

head (Fig. 6). The pigmented growths are well vascularized (Fig. 6, 7) and the epithelium is often thin or sloughed, probably as the result of pressure from the growing tumour. There is some proliferation into adjacent subcutaneous areas, the growing tissue breaking through limiting membranes (Fig. 8) and causing extensive destruction of muscle and bone (Fig. 9).

The cellular elements of the xantho-erythrophoroma are dendritic, rounded or sometimes fusiform in shape. These cells, whatever their shape, are free of pigment as a result of the techniques employed. They are supported by a delicate vascular and connective tissue stroma (Fig. 6, 7, 8, 10, 11). An occasional macromelanophore may be present, particularly around a blood vessel (Fig. 6). No inflammatory reaction is apparent.

The dendritic, rounded and fusiform cells vary in size from 15 to 90 μ at their largest dimensions (Fig. 12). The cytoplasm is finely or coarsely granular (Fig.

7, 8, 10). The nuclei react weakly to staining and may show one or more larger chromatin bodies (Fig. 7, 8, 10, 12). This weak reaction of the nuclei is also noted in Feulgen treated material. Mitotic figures (Fig. 10) as well as degenerative changes are fairly common in these cells. These changes are manifested by vacuolization of the cytoplasm and by the presence of vacuolated, pycnotic, lobulated and fragmented nuclei (Fig. 11). There seems to be no direct relation between the condition of the cytoplasm and that of the nuclei. Cells with either normal nuclei and vacuolated cytoplasm, or with finely granular cytoplasm and

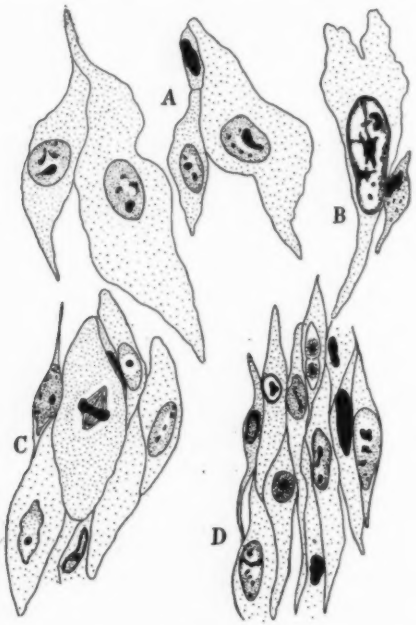

FIG. 17.—Cells from advanced xantho-erythrophoroma (206–11). A–C, Fusiform cells—note mitotic figure in C; D, group of spindle-shaped cells from another region of the tumour showing several stages of pycnosis. A, B and D stained with haemotoxylin-eosin; C with Giemsa's. × 1000.

nuclei in extreme stages of degeneration are found with equal frequency. Cell boundaries are often indistinct and the cells in many instances simulate a syncytium, independently of nuclear changes (Fig. 7, 8, 10, 11). Macrophage activity is sometimes evident around degenerating cells.

In more advanced xantho-erythrophoromas, as represented by Hybrids 206–11 and 206–14 (Gordon, 1950) spindle and fusiform-shaped cells predominate (Fig. 13 to 17). The typical dendritic and rounded cells are relatively fewer in this stage than in the former, and they are usually found at the periphery of the growth (Fig. 16, 18). Only a few melanin-bearing cells are found scattered throughout the tissue. These tumours have a well developed vascular reticulum

and a minimum amount of collagenous fibres, together with numerous nerve fibres, especially in the central regions of the growth. The epithelium is thickened and papillary-like in arrangement (Fig. 18) except in regions where it is sloughed. Mitotic figures are fairly common in the epithelial elements.

The spindle cells in regions of mitotic activity (Fig. 15, 17) measure from 20 to 30 μ in length and from 10 to 15 μ in width. The nuclei contain fine chromatin granules around the periphery and larger, more centrally located chromatin clumps, varying in number and shape (Fig. 17). In other regions of the tumour

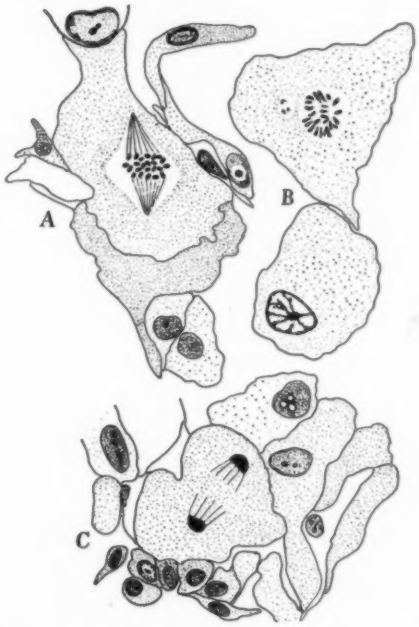

Fig. 22.—Cells from an area in the xantho-erythroporoma free of melanotic cells (243–11). A, Metaphase—note spindle in granule-free area; B, metaphase plate in polar view with a few chromosomes (?) isolated; C, cell in telophase with massed macrophages. Masson's.

FIG. 22, 28, 29, 30, 33 represent different regions of xantho-erythrophoroma (243–11) invaded by melanotic cells. All cells shown here and above were drawn with Camera Lucida at approximately × 1000.

the cells are small and fibroblast-like in appearance (Fig. 13, 14, 17), measuring 15 to 25 μ in length and from 5 to 10 μ in width. In these cells the nuclei are often more elongated; some show median nuclear constrictions (amitosis ?) while others show various stages of pycnosis (Fig. 17). Only a few macrophages, however, are evident in these tumours.

The relatively few dendritic and rounded cells are similar to those described in the simple xantho-erythrophoroma. As was previously mentioned, these are usually present in the periphery of the tumour, but may also be found within

the main mass of the growth, surrounded by the spindle-shaped cells. Occasionally, mitotic figures and binucleate cells are present.

In Hybrids 206–12 (Gordon, 1950) and 243–11, the xantho-erythrophoromas are actively invaded by macromelanophores. The latter infiltrate the red tumour from the posterior and lateral areas of the body. They first spread out along the region immediately below the epidermis, and then inward among the

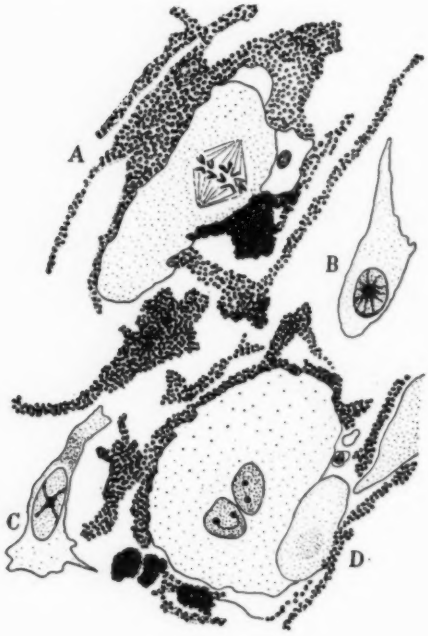

FIG. 28.

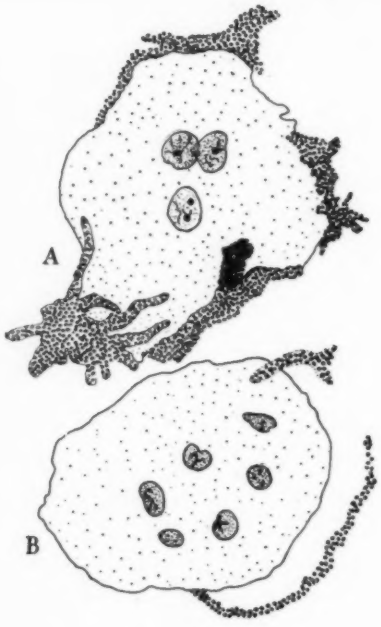

FIG. 29.

Fig. 28.—Cells from region in the xantho-erythrophoroma invaded by macromelanophores (243–11). A, Cell of the xantho-erythrophoroma in metaphase surrounded by macromelanophores; B and C, similar cells isolated to show form and nuclear pattern; D, binucleate cell. The cell in A taken from Masson's stained preparations; others from haematoxylin-eosin.

Fig. 29.—Degenerative changes in cells of the xantho-erythrophoroma (243–11) from area shown in Fig. 22. Masson's stain.

cellular elements of the xantho-erythrophoroma. Eventually, the xantho-erythrophoroma cells degenerate and are more or less completely replaced by melanotic cells. The various stages in the development of the melanoma within the xantho-erythrophoroma can be demonstrated and traced in one of these fishes (243–11). The most anterior portion of the growth shows a histological picture similar to the pure xantho-erythrophoroma described above, with dendritic and rounded cells predominating (Fig. 19). The cytoplasm of some of these xantho-erythrophores contains coarse granules which stain a brownish-grey with

haematoxylin-eosin and which, at first glance, may be mistaken for melanin bodies (Fig. 20). With Giemsa's method, however, they take on a purplish colour, whereas the melanin granules are stained a blue-green. These purplish coloured granules are also not to be interpreted as inclusion bodies of the type usually associated with virus infections.

The nuclei of the xantho-erythrophores in the advanced red tumours are similar in form and structure to those seen in the cells of the simple xantho-erythrophoroma. However, mitotic figures and binucleate cells (Fig. 21, 22) are

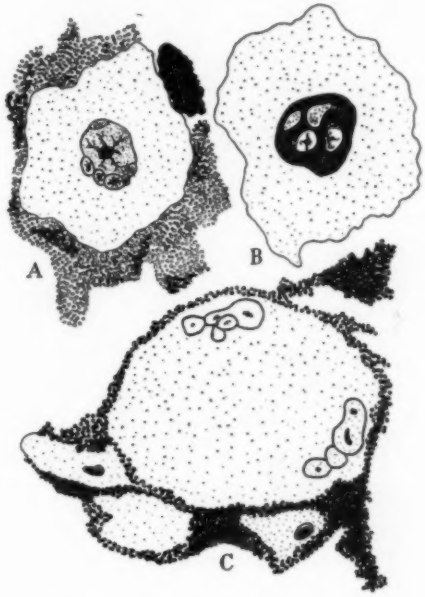

Fig. 30.—Degenerative changes in tumour (243–11), same region as Fig. 22 and 28. Haematoxylin-eosin.

found more frequently in the invaded red tumour. In regions of degeneration, macrophages are common (Fig. 22, 23), many of which, in haematoxylin-eosin preparations, are seen to be filled with yellowish, irregularly shaped refractive bodies (haemosiderin ?) ; others are filled with brownish granules, which, in Giemsa's preparations, appear purplish and which are somewhat similar to the cytoplasmic granules described above. The few macromelanophores present in this area are usually associated with blood vessels (Fig. 19).

In other regions of these invaded red tumours, the sub-epidermal macromelanophores grow inward (Fig. 24), filling the interstitial spaces. These highly dendritic cells surround the cells of the xantho-erythrophoroma (Fig. 24 to 27, 28 to 30), many of the latter showing degenerative changes (Fig. 28 to 30). Mitotic stages and binucleate cells, however, are frequently found (Fig. 26, 28). This

region also shows a few melanin-bearing macrophages and comparatively large numbers of macrophages without melanin.

In its posterior and ventral regions the invaded tumour exhibits characteristics of a melanoma (Fig. 31, 32). The growth in these regions is composed chiefly of melanotic cells, supported by a rich vascular and connective tissue stroma. Numerous macrophages with and without melanin, are also present (Fig. 33). Occasionally remnants of xantho-erythrophoroma cells, or only their

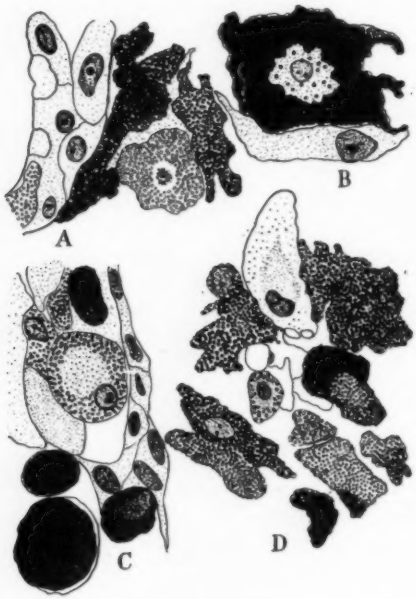

Fig. 33.—Melanotic area of the tumour (243–11). In this region one or two cells from the original xantho-erythrophoroma can still be distinguished. In other areas, however, they are completely absent. A, Region just below the epidermis—note the melanotic cell (melanoblast) in metaphase; B, typical melanotic cell of this region associated with a cell of the xantho-erythrophoroma; C, group of melantotic cells just below the epidermis showing different patterns; D, a similar group around lacunae together with a melanin-laden macrophage. Masson's.

nuclei, are found. The melanotic cells (melanoblasts) are smaller and less dendritic than the typical macromelanophores; they are usually rounded, sometimes fusiform, varying from 10 to 40 μ at their widest dimensions. Although melanin granules of apparently similar size are present in all these cells, the cells show differences in coloration, depending on the intensity and the distribution of granules within the cytoplasm. In Feulgen preparations the melanin granules of these fish tumours retain their characteristic colour, thus differing from the Harding-Passey mouse melanoma in that the melanin granules of the latter are partially bleached by this process.

Nuclear structure and division stages are observed in melanoma cells in which

melanin granules are amber and more evenly distributed (Fig. 33). As in typical melanomas of other platyfish-swordtail hybrids, these melanotic cells show invasive characteristics, with extensive destruction of adjacent muscle, bone, scales, sense organs, and in addition the tissues of the xantho-erythrophoroma.

DISCUSSION.

The abnormal growths described above were originally reported as mixed pigmented tumours and referred to as erythromelanomas. The present studies, however, show that two distinct tumours are involved : a xantho-erythrophoroma in the head region and a melanoma of the trunk and caudal peduncle. In some of the hybrids the more actively proliferating cells of the melanoma invade and replace the red pigmented tumours of the head, producing an erythromelanoma.

The red and yellow pigment cell characteristic of the xantho-erythrophoroma was first described by Kosswig (1928) from normal fish as an *erythro-xanthophore*. According to Goodrich, Hill and Arrick (1941), the cell, which they called a *xantho-erythrophore*, is a xanthophore in which erythropterin is laid down in addition to lutein and zeaxanthin.

In fixed and stained tumorous material the xantho-erythrophores are either non-pigmented dendritic, rounded or fusiform cells. In both normal and tumorous tissues the nuclei of these cells are weak in chromatin ; nevertheless, mitotic figures are frequently found. The mitotic divisions appear normal in all instances. There is no indication of either reduction of chromosome number or polyploidy, as was reported for cells of melanomas in fishes by Breider (1939) and Levine (1948). Precession and lagging of chromosomes at anaphase have not been found in our material.

The nuclear and cytoplasmic degenerative changes here described are similar to those reported by Ermin (1946) in red, black-spotted (*RSp*) platyfish-swordtail hybrids. In our series of tumours, however, these changes, associated with macrophage activity, are more striking in xantho-erythrophores which are surrounded by melanotic cells. The degeneration of the xantho-erythrophoroma probably results from the growth-pressure of the apparently more actively reproducing melanotic cells which infiltrate and penetrate the interstices between the xantho-erythrophores. Even under such conditions binucleate xantho-erythrophores were found.

In regions of heavier concentration of melanotic cells, however, degeneration of the cellular elements of the xantho-erythrophoroma was more or less complete. Here the melanotic cells show evidence of considerable reproductive activity, with proliferation even into the surrounding normal tissues. Mitotic figures are seen in these cells.

These melanotic cells are not modified macrophages, and are apparently not comparable to the pigment cells derived from macrophages reported by Niu and Twitty (1950) in transplanted salamander skin. Nor is there any indication of an " infective " process such as was reported by Billingham and Medawar (1948) for the transformation of non-pigmented dendritic cells into melanin-bearing ones in guinea-pig skin.

The invasion of one neoplastic growth by another is exceptionally rare. In a personal communication Dr. A. P. Stout stated that he has observed a melanoma of the human choroid which metastasized and grew in the wall of an ovarian

cystadenoma, and another melanoma which grew in a leiomyoma of the uterus. This problem was discussed with other pathologists, and several cases were mentioned in which neoplastic tissues other than melanoma were the invading elements. In all cases, however, the neoplastic tissues in these growths were still distinguishable. Whether or not the metastasizing tissue would have completely replaced the invaded tumour was not determined. Although complete replacement of a xantho-erythrophoroma by melanoma has never been observed in our fishes, the evidence seems to indicate that this would have occurred had the specimens lived longer.

An interpretation of the nature of the genic action that modifies pigment cell growth and the physiological effects of the interaction of such genes in platyfish-swordtail hybrids was outlined by Gordon (1948, 1950). The effect of the various genes for pigment cells upon the development of the red, the black and the red-black pigment cell tumours may now be suggested.

In the normal platyfish Gordon (1928) showed that the dominant, sex-linked, spot-sided gene, *Sp*, manifests itself in the development of large black pigment cells or macromelanophores; the large melanophores first appear in post-embryonic platyfish in the posterior region of the body, specifically, in the caudal peduncle. As the fish grows to maturity the macromelanophores increase in number in the corial tissues and spread toward the head. The number of macromelanophores produced by the *Sp* gene in the normal, spotted platyfish is influenced strongly by the action of the stippled gene, *St*, a dominant, autosomal factor for the presence of many micromelanophores. When *Sp* is associated with the dominant gene *St* (that is, *Sp St*), more macromelanophores develop than when the *Sp* gene is associated with the recessive gene *st* (that is, *Sp st*). Furthermore, in both the *Sp st* post-embryonic and adult platyfish the macromelanophores are restricted for the most part to the caudal peduncle. The inter-

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EXPLANATION OF PLATES.

FIG. 2.—Living, typical, highly dendritic xantho-erythrophore from scale of domesticated red swordtail. The granules are red and the clear cytoplasmic area is yellow. × 500.

FIG. 3.—Piece of biopsied skin removed from hybrid fish (286) with xantho-erythrophoroma; the numerous round cells contain pigment similar to the cell shown in Fig. 2. × 125.

FIG. 4.—Edge of biopsied tissue, shown in Fig. 3, at a high magnification. Note the elongated macromelanophore. × 250.

FIG. 6 to 11 are photomicrographs taken from an early xantho-erythrophoroma as seen in hybrids (243–12 and 243–13). In such tumours the few macromelanophores present are usually found around blood-vessels.

Fig. 6.—A typical area of a section through an early xantho-erythrophoroma. These growths are localized in the corium. Masson's. × 125.

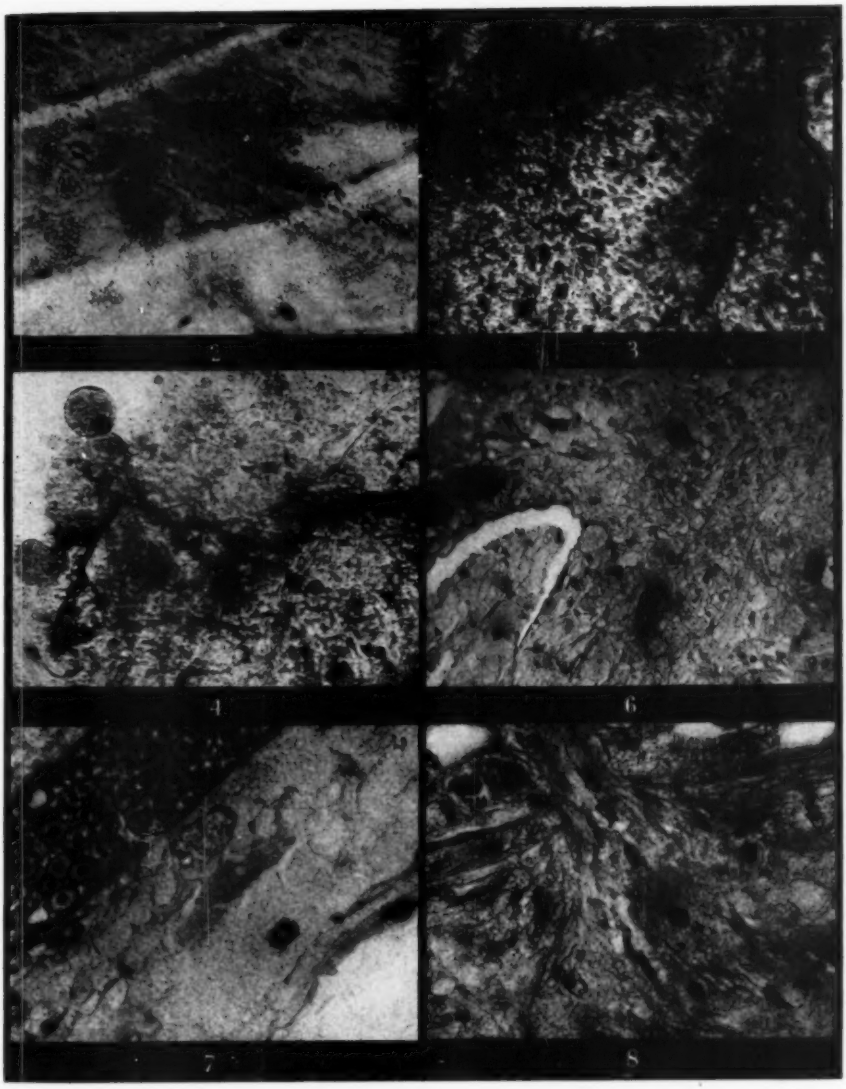
Fig. 7.—Details of cells shown in Fig. 6. Note the indistinct cell boundaries. Masson's. × 1125.

Fig. 8.—Same tumour as in Fig. 6 and 7. The tissue shows considerable proliferative activity. Masson's. × 1125.

Fig. 9.—Xantho-erythrophoroma showing extensive destruction of surrounding tissues. Masson's. × 45.

Fig. 10.—Region of xantho-erythrophoroma showing nuclear details; note the telophase stage. Masson's. × 1350.

Fig. 11.—Cells of the xantho-erythrophoroma showing cytoplasmic and nuclear degenerative changes. Note indistinct cell boundaries, vacuolated cytoplasm and cell with greatly lobulated nucleus. Masson's. × 1350.



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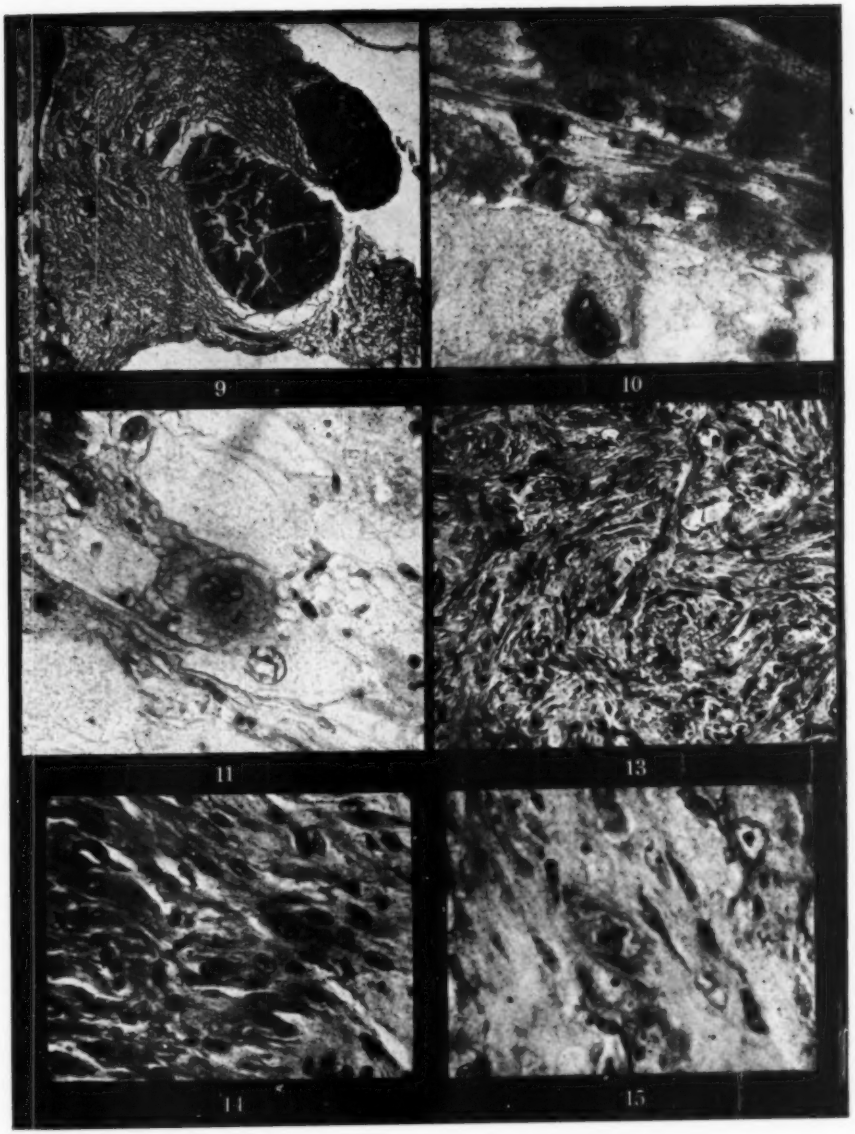

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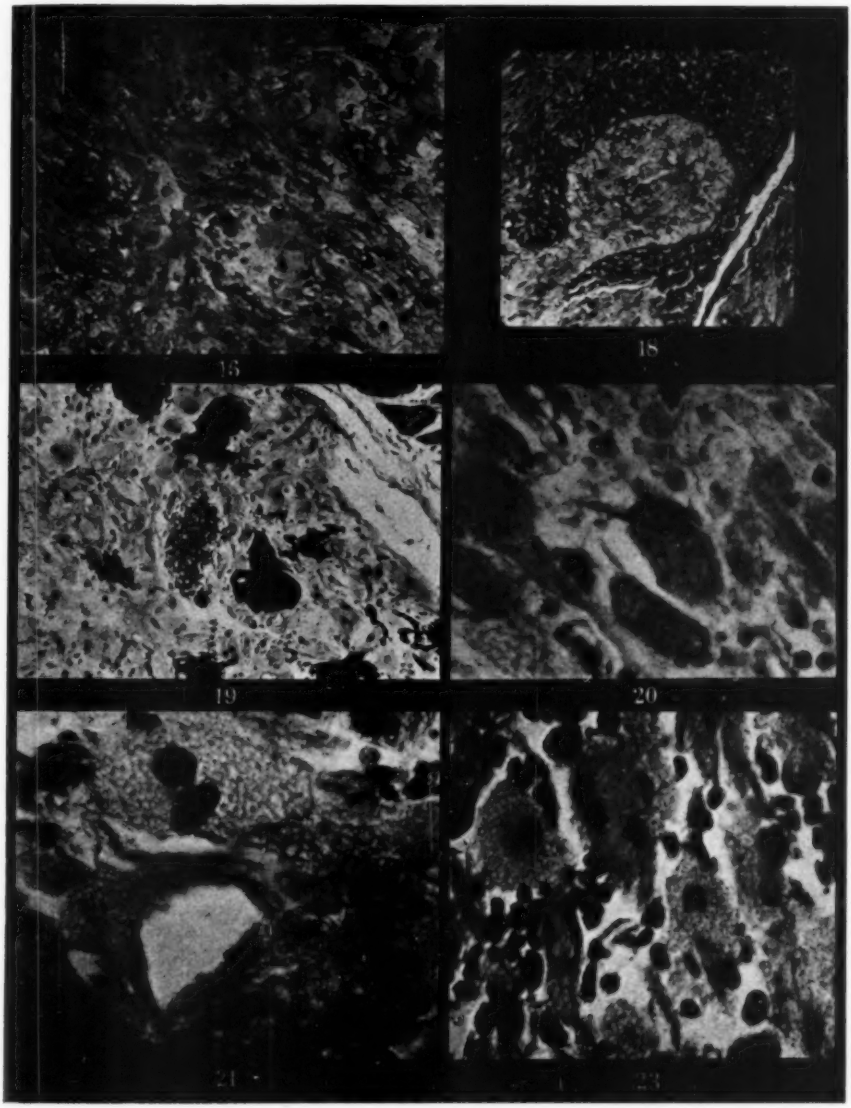

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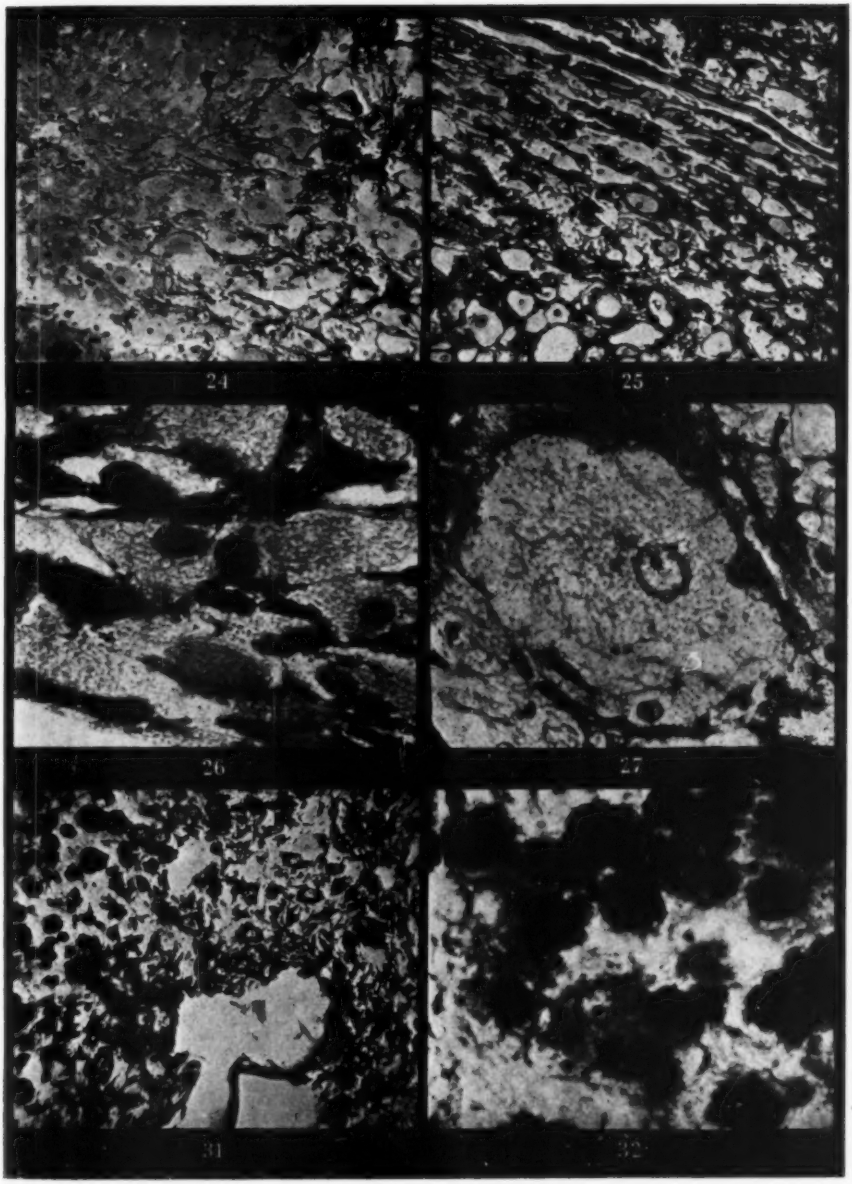

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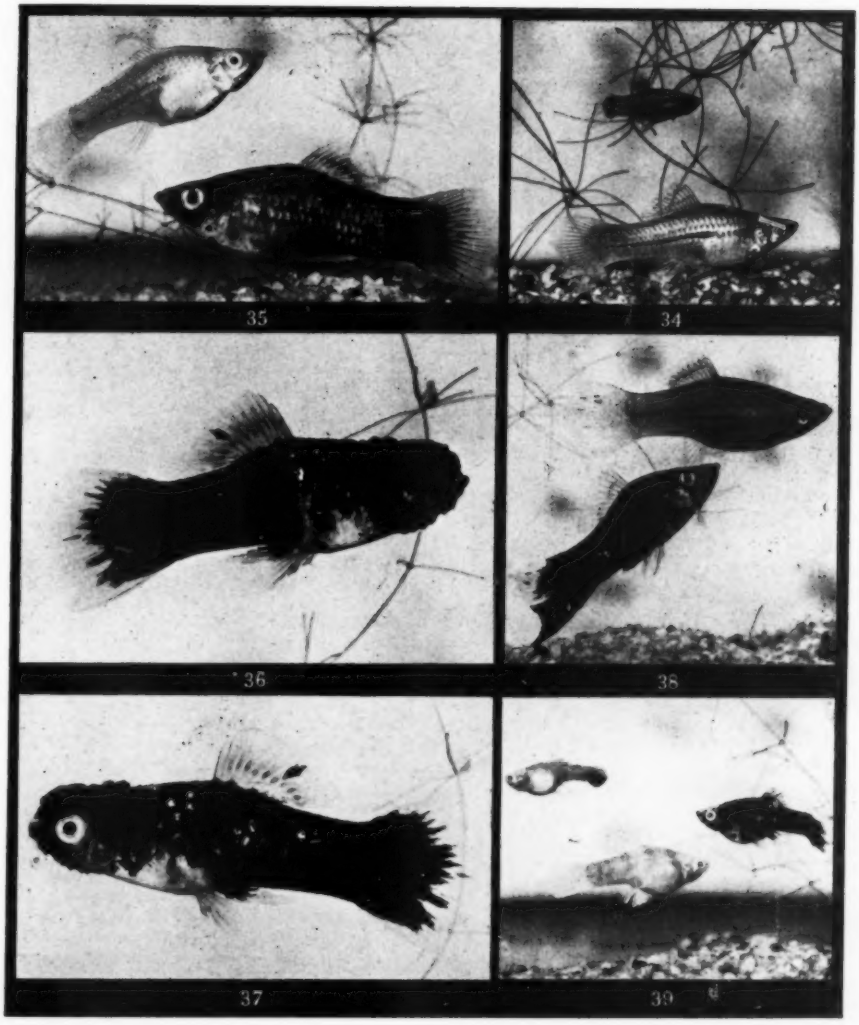

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FIG. 13 to 16, 18 are photomicrographs of sections from an advanced tumour, as found in hybrids (206–11 and 206–14), composed of smaller but typical cellular elements of the xantho-erythrophoroma and numerous fibroblast-like cells. Only a relatively few melanin-bearing cells are associated with these tumours.

Fig. 13.—Fibroblast-like area of the tumour. Masson's. × 315.

Fig. 14.—Details of region shown in Fig. 13. Masson's. × 1200.

Fig. 15.—Spindle-shaped cell in mitosis in advanced xantho-erythrophoroma. Giemsa's. × 600.

Fig. 16.—Region of the tumour showing more or less typical dendritic and rounded cells. Masson's. × 260.

Fig. 18.—Papillary arrangement of the thickened epithelium overlying the growth shown in Fig. 16. Masson's. × 150.

FIG. 19 to 21, 23 to 27, 31, 32 are photomicrographs of xantho-erythrophoroma which show successive stages of invasion with melanotic cells, as seen in hybrids (206–12 and 243–11).

Fig. 19.—Region from the anterior part of the tumour showing typical elements of the xantho-erythrophoroma and several macromelanophores. Masson's. × 125.

Fig. 20.—Giemsa-stained section from the same region shown in Fig. 19, demonstrating cells with differences in cytoplasmic granulation. Note the macrophage. Giemsa's. × 1125.

Fig. 21.—Cells from the same region showing a binucleate stage and a mitotic figure (metaphase), commonly encountered in this tumour. Masson's. × 1125.

Fig. 23.—Another region of the same tumour as that in Fig. 19 to 21, showing numerous macrophages around degenerating cells of the xantho-erythrophoroma. Masson's. × 1125.

Fig. 24.—Low power view showing the infiltration of sub-epidermal macromelanophores among the cells of the xantho-erythrophoroma. Masson's. × 275.

Fig. 25.—Area of the tumour showing a more extensive infiltration with melanotic cells. Masson's. × 275.

Fig. 26.—Higher magnification of Fig. 25 to show detail of the cellular elements. The melanotic cells occupy the interstitial areas of the tumour. Masson's. × 1100.

Fig. 27.—Considerably enlarged cell of the xantho-erythrophoroma surrounded by a highly dendritic melanotic cell. Iron-haematoxylin. × 550.

Fig. 31.—Posterior-ventral part of the tumour characterized by a complete invasion of melanotic cells, only a few of the elements of the original xantho-erythrophoroma remaining. Masson's. × 140.

Fig. 32.—Details of the tissue in the region shown in Fig. 31. × 1240.

FIG. 34.—Above, a red-throated, spot-sided male platyfish, *RtSp st*, pedigree No. 173–15; below, an albino swordtail, *++ stii*, pedigree No. 182–1. These two are normal parents, yet they produced a strain of red tumorous, black tumorous and red-black tumorous hybrids, pedigree No. 206.

FIG. 35.—Below, a platyfish-swordtail hybrid of the genetic constitution *RtSp*, 206–15, showing the early stage of the development of the xantho-erythrophoroma. This shows up as a red patch on the left operculum surrounding the lower half of the eye. The early stages of melanosis appear on the caudal peduncle. Above, an albino swordtail, 182^2–2.

FIG. 36, 37.—A right and left view of a platyfish-swordtail hybrid, 206–11, having the genetic constitution of *RtSp*, showing a large erythromelanoma of the head and a melanosis in the caudal area. In this tumour the melanoma cells have invaded, destroyed and replaced some of the erythrophoroma cells.

FIG. 38.—Below, an *RtSp* platyfish-swordtail hybrid, 206–16, that has a melanoma of the tail and general mid-ventral area, but no red tumour of the head; above, a female sibling, 206–5, a normal hybrid, recessive for the red and black colour genes.

FIG. 39.—Three offspring of pedigree No. 241, produced by mating a platyfish-swordtail *RtSp* hybrid, 206–12, exhibiting an erythromelanoma, back to an albino swordtail, 182^2–6; upper left, an albino with an amelanotic melanoma at the tip of a caudal fin; lower left, a sibling showing the "ghost" pattern of macromelanophores; right, typical melanotic hybrid showing the early stage of melanosis in the posterior region of the body. None of these fishes developed an erythrophoroma.

action of *Sp* and *st* is also observed in the *RtSp st* and in the *DrSp st* platyfish. *Rt* and *Dr*, it should be noted, control red pigment cells.

Essentially the same early developmental stages in the growth of macromelanophores are observed in the *RtSp st* platyfish-swordtail hybrids, produced by mating a ruby-throated, spot-sided, stippleless platyfish and an albino swordtail (Fig. 34). These are the hybrids in which melanomas, erythromelanomas, and xantho-erythrophoromas may develop. In the post-embryonic *RtSp st* hybrids the macromelanophores first appear in the caudal peduncle and then gradually spread, at least in part, by cell multiplication to the anterior regions (Gordon, 1950).

The effects of the ruby-throated gene, *Rt*, are also quite striking. In the young of *RtSp st* platyfish-swordtail hybrids xantho-erythrophores developed in the opercular region. The bright red coloration is produced by the action of the *Rt* gene of the platyfish in association with *Rt*-modifying genes of the swordtail (Gordon, 1950).

Thus in the early stages of development of the *RtSp st* platyfish-swordtail hybrids, the *Rt* gene and its modifiers influence the growth of red pigment cells in the anterior part of the body, while the *Sp* gene and its modifiers determine the growth of macromelanophores in the posterior regions.

As the *RtSp st* hybrids develop, the red pigment cells produce a *xantho-erythrophoroma* in the opercular area, involving the entire head in some individuals (Fig. 35). At the same time the macromelanophores produce a state of melanosis in the caudal peduncle region. By a process of extension of atypical cell growth—mainly in the corium and, to a lesser degree, in the subcutaneous tissues—the macromelanophores and the melanoblasts produce a *melanoma* and invade the anterior parts of the body. These two centres of atypical cell growth, the red and the black, eventually produce a number of results. In some hybrids the black tumour cells reach, penetrate, destroy and replace the red tumour cells and so produce an *erythromelanoma* (Fig. 36, 37). In other hybrids the xantho-erythrophoroma of the head remains uninvaded because the macromelanophores do not reach the head region. In still other hybrids the macromelanophores apparently grow too rapidly and overrun the head region before the red pigment cells produce a red tumour in that area (Fig. 38). This means that a state of general melanosis of the entire body may prevent the development of a xantho-erythrophoroma. Thus it may be seen that the genetic control of these tumours in hybrids, all of which carry the *RtSp st* genes, is not absolutely rigid (Fig. 39).

Other investigators have reported red and red-black tumours in platyfish-swordtail hybrids not carrying *RtSp st* genes. For example, Kosswig (1929, 1931) reported an erythrophoroma in a platyfish-swordtail hybrid carrying the genes *RSp* and in one carrying *DrSr*. His symbol for *DrSr* was *Mo*. Ermin (1946) studied one of Kosswig's *RSp* tumorous hybrids, and this has been discussed elsewhere in this paper. Finally, Smith, Coates and Strong (1936) described an erythrophoroma in the dorsal fin of a *Dr* hybrid. In the course of his genetic studies Gordon (1950) found only one erythromelanoma in hundreds of red-bodied, black-spotted *RSp* hybrids, and one in a red-finned, stripe-sided *DrSr* hybrid. These rare instances are evidence that similar phenotypes may have different genetic constitutions.

In his discussion of the genetic origin of erythromelanomas, Gordon (1950) indicated that even though the genetic constitution of the parents of a platyfish-

swordtail hybrid were known in terms of genes, conditions in the offspring could not be predicted with complete assurance unless the spacial relationship of those genes were also known. The minimum requirement for the production of the erythromelanoma in platyfish-swordtail hybrids is the presence of the linked genes *RtSp*. Gordon (1950) showed that a platyfish parent of the genetic constitution of *RtSb/DrSp* would not, while a *RtSp/DrSb* would produce some hybrids with erythromelanomas when mated with an albino swordtail. The genes, *Rt* and *Sp*, must be linked on the same chromosome, for only in this arrangement are they carried in the same gamete. These details indicate the preciseness of the genetic (chromosomal) control of pigment cell development. They also show the importance of studying the various genes and gene combinations and their specific developmental effects upon the normal and atypical growth of specific cells.

SUMMARY.

1. The histology and cytology of xantho-erythrophoromas and xantho-erythrophoromas invaded by a melanoma are described in a genetically related group of platyfish-swordtail hybrids.

2. The main cellular elements in these tumours are xantho-erythrophores, which contain both red (erythropterin) and yellow (lutein and zeaxanthin) pigment and macromelanophores, large cells containing melanin.

3. In the tumorous animals the xantho-erythrophoroma develops rapidly in the head region, and at the same time a condition of melanosis develops in the posterior region of the body. In some individuals the melanotic cells show great reproductive activity and produce a melanoma. In those animals that develop a melanoma the cells of the melanoma may invade the xantho-erythrophoroma and destroy and replace the cells of the red pigmented tumour. In other cases the melanin-bearing cells are restricted to the posterior part of the body, allowing the red tumours to develop on the head without the involvement of the black pigment cells. The xantho-erythrophoroma and melanoma also invade and destroy the normal surrounding muscle, bone and connective tissue.

4. The sequence of events in the invasion of one kind of neoplastic tissue by another type is discussed from the points of view of histology and developmental genetics.

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REFERENCES.

BILLINGHAM, R. E., AND MEDAWAR, P. B.—(1948) *Heredity*, **2**, 29.
BREIDER, H.—(1939) *Z. wiss. Zool.*, **152**, 89.
ERMIN, R.—(1946) *Rev. Fac. Sci. Univ. Istanbul*, Ser. B, **11**, 147.

GOODRICH, H. B., HILL, G. A., AND ARRICK, M. S.—(1941) *Genetics*, **26**, 573.
GORDON, M.—(1928) *J. Hered.*, **19**, 551.—(1948) 'The Biology of Melanomas,' *Spec. Publ. N.Y. Acad. Sci.* (4), 216.—(1950) *Endeavour*, **9**, 26.
Idem AND NIGRELLI, R. F.—(1950) *Cancer Res.*, **10**, 220.
Idem AND G. M. SMITH.—(1938) *Amer. J. Cancer*, **34**, 255.
KOSSWIG, C.—(1928) *Z. indukt. Abstamm.- u. VererbLehre*, **47**, 150.—(1929) *Ibid.*, **52**, 114.—(1931) *Ibid.*, **59**, 61.
LEVINE, M.—(1948) 'The Biology of Melanomas,' *Spec. Publ. N.Y. Acad. Sci.* (4), 177.
NIGRELLI, R. F., JAKOWSKA, SOPHIE, AND GORDON, M.—(1950) *Cancer Res.*, **10**, 234.
NIU, M. C., AND TWITTY, V. C.—(1950) *J. exp. Zool.*, **113**, 633.
SMITH, G. M., COATES, C. W., AND STRONG, L. C.—(1936) *Zoologica, N.Y.*, **21**, 219.

SOME UNUSUAL GONADAL TUMOURS OF THE FOWL.

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NEOPLASMS involving the ovary are common in the fowl (Olson and Bullis, 1942 ; Campbell, 1945). The great majority fall into one or other of two categories, namely adenocarcinomatous growths in various stages of differentiation from the frankly anaplastic type to the sclerosing tumour, or, secondly, lymphomatous growths associated with the leucotic complex, e.g., lymphocytoma, fowl paralysis tumour, aleukaemic lymphoid leucosis, etc. Most of these lymphoid growths should not be classified as ovarian tumours, since in view of the generalized nature of the leucotic process it is usually impossible to be certain whether they are primary in that organ or not. They are mentioned simply because of the frequent reference in avian pathological literature to "lymphoid ovarian tumours."

In human pathology, besides the common adenocarcinomatous types of ovarian tumour, several other rarer forms are recognized as follows : Brenner tumour (oöphoroma folliculare) : granulosa-cell, thecal, and luteal tumours ; arrhenoblastomas ; dysgerminomas.

A survey of the literature dealing with neoplastic disease of the fowl shows the apparent extreme rarity of such tumours. Seifried (1923) recorded a Brenner tumour of the fowl and compared it with its human counterpart, and Friedgood and Uotila (1941) detailed 5 cases of ovarian "tumours" associated with virilism in the fowl. In two of these latter cases, tuberculosis of the ovary complicated the picture, and the remainder were cystic or contained small growths which appeared to be tumours in various stages of degeneration. Tentative diagnoses of arrhenoblastoma were made, but it was also suggested that they might be luteal-cell tumours or "hypernephromata." The only record of an ovarian teratoma in the fowl is by Jackson (1936).

With regard to the male bird, there appear to be no reports of spontaneous seminoma or interstitial-cell tumour, but several cases of spontaneous teratoma testis have been recorded (Sheather, 1911 ; Jackson, 1936 ; Olson and Bullis, 1942).

It seemed reasonable to believe that the absence of reports of the rarer forms of ovarian and testicular tumours was simply an indication of a lack of intensive study in this branch of comparative pathology. It was therefore determined to examine as many tumours arising in these sites as possible. To date approximately 2000 cases have been submitted to a thorough post-mortem and histological scrutiny, and, as was anticipated, several hitherto unrecorded types of tumour of the fowl gonads have been encountered.

It is desirable that an account of these should be put on record, not only because of their interest from the viewpoint of comparative oncology, but also

because the study of such tumours may throw a light on the origin of their human counterparts, and may help to settle the much debated questions of inter-relationship and correlation between histological structure and endocrinological function.

METHODS.

All the tumours described in this paper arose spontaneously. Some of the birds came to autopsy without any history. Others were obtained alive with a history of unusual behaviour such as the development of male traits in the hen, cessation of laying, enlarging abdomen, etc. At post-mortem a thorough search was made for metastases, and the possibility that the gonadal tumour was not the primary growth was ruled out as far as possible. Blocks of tissue were taken from many organs for histological examination, whether they showed visible abnormality or not. They were fixed in formal-saline, Susa or Helly, embedded in paraffin wax and cut at 8μ, and were stained by a variety of methods. Frozen sections were studied when the occasion permitted. Photographs of gross specimens were taken in many cases.

PATHOLOGY.

Tumours of the Female Gonad.

Tumours of the female gonads will be dealt with first.

(1) This tumour was encountered in a Rhode Island Red hen in its third year. Upon cessation of laying the bird was killed for table purposes, and the carcase was brought to the laboratory upon the same day.

The ovary was inactive, and a large part of it was replaced by a firm, faintly yellow and smoothly bosselated tumour 4·0 × 3·5 × 3·8 cm. in dimension. A few small cysts were visible on the surface, and a larger cyst filled with a brownish mucoid fluid occupied the caudal pole of the mass. The growth offered some resistance to the knife, and its cut surface showed pale yellow fibrous areas containing numerous small cysts and islands of pinkish tissue. There were no implantations in the abdominal cavity and no metastases were found.

Histologically a large part of the tumour consists of a stroma of interlacing bands and whorls of fibrous tissue, mostly dense, but having a looser texture in some places. There is a certain amount of plain muscle (characteristic of the avian ovary especially at the hilus) in one part of a section. Embedded in the dense fibrous tissue are numerous epithelial-lined cysts of varying size, some exhibiting a single layer of flattened cells, others lined by several layers, the innermost of which are typical columnar mucous secreting cells (Fig. 1). No cilia are detectable. The lumen of many cysts contains a granular eosinophilic material. Scattered between these structures are a few solid nests of undifferentiated epithelial cells with large pale nuclei. These show a tendency to develop a lumen (Fig. 2).

In view of its undoubted ovarian origin, its benign character and its strikingly similar structure to the Brenner tumour of the human subject, this case—the only one of its kind in the whole series of ovarian tumours so far examined—was classified as such. According to Willis (1948) it is probable that Brenner tumours arise in the human from nests of cells first described by Walthard in 1903. These may be found on the surface of the ovary, Fallopian tube or broad ligament. At the same time he points out that no extra-ovarian Brenner tumour has ever been

reported, which, in view of the distribution of Walthard nests, raises the question of the validity of this etiological theory.

In an effort to establish whether comparable " nests " are present in the fowl, a considerable number of ovaries, oviducts and dorsal ligaments have been examined both by naked eye and histologically, with negative results. Such a finding of course does not rule out their existence in the fowl, but it does indicate that should other workers fail to find them, an alternative explanation will have to be considered. It may be relevant to indicate here that in the hilus of the fowl's ovary and in the oviduct ligament, a vestigial structure is to be found representing a rudiment of the genital part of the mesonephros and corresponding to the epididymis of the male. A comparison of this structure (Fig. 3) known as the parovarium, with the tumour described above shows a great similarity between the two. A comparable structure occurs in the human subject, and is well illustrated by Nicholson (1950).

(2) The next ovarian tumour to be considered is the granulosa-cell type, of which 4 cases have been studied in this series. They all occurred in adult hens, namely 2 Buff Rocks and 2 crossbreds. Unfortunately none of these was seen alive. In 2 cases there were extensive implantations from multiple ovarian tumours (Fig. 4) to the serous surface of the gut, oviduct, liver and kidney, and in one instance a metastatic growth was found in the lung. In the other 2 cases, the tumours were found at the site of the rudimentary right gonad, and one was associated with a small persistent cystic right oviduct. In both cases the left functional ovary appeared normal.

The gross appearance of these tumours varied somewhat. In general they were fairly firm, white, or with yellowish areas, sometimes cystic and occasionally haemorrhagic. It was interesting to note that in the first 2 cases mentioned above, although the ovary was not functional, the oviduct had the appearance and was nearly the size of an active organ.

Histologically, these tumours are characterized by a quite variable structure in different regions or even in different areas of the same section. One case, for example, shows mainly a " cylindroid " type of growth, with cords of polygonal epithelial cells with large clear nuclei and one or two prominent acidophilic nucleoli, lying in a fine connective tissue stroma (Fig. 5). A folliculoid formation is common in some areas, and the stroma between these groups of cells shows hyalinization (Fig. 6). Other cases show a more undifferentiated structure, but still a tendency to form " follicles," and in one case a very marked luteinization of the granulosa cells was present (Fig. 7). So noticeable is this feature in certain areas that a diagnosis of luteoma would be justifiable. " Typical " rosettes as seen in human tumours, characterized by a stellate arrangement of cells around a cystic space containing fluid or a degenerating cell, apparently do not occur in avian granulosa-cell tumours.

(3) One example of a theca-cell tumour was found at autopsy in a Buff Rock hen in her second year. It consisted of a yellowish irregular nodular mass measuring $5 \times 6 \times 4{\cdot}5$ cm. (Fig. 8). It was of a soft consistency and the exterior tended to be friable. On section, it was cream to yellowish in colour and displayed bands of tissue which had a tendency to radiate from the centre of the growth. The vascularity appeared to be very poor. The rest of the ovary seemed normal, with a few atretic follicles, and the oviduct was that of a bird in full lay. Histologically, this tumour is mainly composed of round oval or fusiform cells, with a

fine chromatin network in the nucleus and a rather foamy cytoplasm (Fig. 9). Mitosis is extremely rare. In some regions, particularly near the periphery, these cells become polyhedral, with round nuclei and vacuolated cytoplasm. Frozen sections stained with Sudan IV show abundant lipoid material in such cells. The periphery of the growth is largely necrotic. Radiating bands of smooth muscle occur in the interior of the section.

Willis (1948) states that ovarian fibromata are probably the end result of a theca-cell tumour. In that case, the rarity of the latter in fowls may account for the equally rare occurrence of fibrous tissue tumours associated with this organ. As an instance of this the only case of ovarian fibroma encountered in this series is worth mentioning. It was found in a Rhode Island Red hen in her third year, and weighed 570 g. including the small inactive ovary (Fig. 10).

(4) Glynn (1921) states that there is no evidence (in the human subject) that heterotopic adrenal cortical tumours arise in the ovary. Willis (1948) agrees, and adds that no acceptable report exists showing the presence of accessory adrenal tissue in the ovary, and he concludes with Glynn that " these growths are all luteinized ovarian tumours." Later, however (p. 510), Willis admits the possibility of very rare adrenal cortical tumours primary in the ovary. Maximow and Bloom (1948) state that what are usually considered chromaffin cells (sympathicotrophic cells) are to be found at the hilus of the human ovary. These apparently have much in common with the medullary cells of the adrenal, although they cautiously conclude that until these cells have been shown to elaborate epinephrin, their relationship to adrenal medulla must remain unproven. The chromaffin reaction of such ovarian cells is unreliable but this also applies to the adrenal medullary cells, where the staining by chrome salts may vary from practically none to a very deep brown.

As far as can be ascertained there is no record of chromaffin cells or heterotopic adrenal cortical tissue in the avian ovary. That such cells may in fact be occasionally present seems to be implied by the following cases, and a thorough cytological examination of the region of the hilus should clear up this point.

The first of these tumours to be described occurred in a Brown Leghorn hen which was brought for examination after developing signs of masculinization. The bird was said to have ceased laying, commenced crowing and to have endeavoured to copulate with other hens in the run. The comb was decidedly male in character, and plumage changes were evident especially in the neck and tail.

Upon post-mortem, a rounded soft yellow-brown tumour measuring 3·8 × 3·5 × 2·8 cm. was found in the ovary, which was inactive. The oviduct was atrophied. Both adrenals were intact and were removed with other organs for examination.

Histologically the tumour has a distinct capsule. Numerous irregular trabeculae containing vessels arise from this and pass into the interior of the growth. Between these are solid islands of large polyhedral epithelial cells which are in turn divided into small irregular groups by fine connective tissue staining as for reticulum. The cytoplasm is eosinophilic and faintly granular. In places it is vacuolated. The nuclei are spherical and fairly constant in size, being situated usually in the centre of the cell. They have a finely scattered chromatin content and one or two inconspicuous nucleoli. There are no mitotic figures (Fig. 11).

This case is an excellent example of the pitfalls of diagnosis based upon purely

morphological grounds, especially when applied to gonadal neoplasms. Superficially the tumour may be thought to resemble a luteoma, but the secretion of androgens weighs against this. It is of interest however to compare it with a small group of thecal luteal cells in the fowl's ovary (Fig. 12). A " corpus luteum " derived from the granulosa cells presents an entirely different picture (Fig. 13).

Other possible explanations are two in number. Firstly the growth may represent a heterotopic adrenal cortical tumour, which Ewing (1942) states to be highly masculinizing but otherwise difficult to differentiate from luteomas. The views of Willis (1948) on the subject have already been stated. Or secondly it may be a case of arrhenoma with such advanced luteinization (Fig. 16) that it bears no resemblance to " text-book " illustrations of that tumour.

It may be stated here that Meyer (1931) concludes that there is hardly any ovarian tumour pattern which cannot be reproduced in the arrhenomata. This view is substantially supported by Burrows (1943). If this is the case, then obviously ovarian tumours can no longer be classified according to their histology but only by their endocrine effects, if any, on the host.

What are we to conclude ? A comparison of the histology of this tumour with normal adrenal cortical tissue of the fowl shows a striking resemblance (Fig. 14). Silver preparations for reticulum also resemble the pattern found in the adrenal cortex. Measurements of thecal luteal cells in the ovary, adrenal cortical cells, and those in the tumour under discussion show that the last two have a similar range in size, whereas the thecal cells are smaller (thecal luteal cells 11·2μ, nucleus 3·2μ, adrenal cortical cells 16·0μ, nucleus 4·8μ, and tumour cells 19·2μ, nucleus 4·8μ, all measurements averages from fixed cells.)

It is now generally agreed that morphological resemblance does not necessarily imply physiological identity of function. Indeed Willis (1948) goes so far as to say " It would not be surprising if further research should show that the two classes of tumour (œstrogenic and androgenic) are really one, and both derived from the ovarian parenchyma." If this should prove to be the case, the naming of such tumours on morphological grounds cannot be justified, and they should be classified according to their endocrine activity.

This tumour was associated with virilism, and is therefore presumably a heterotopic adrenal cortical cell growth or an arrhenoma. The first view is favoured as being the more probable.

(5) The next case, though not a masculinizing growth, is worth recording for its unusual nature, and because its interpretation could mean that besides adrenal cortical cells, chromaffin cells resembling those of the adrenal medulla may occur in the fowl's ovary and may undergo neoplasia in common with other heterotopic tissues.

During the examination of a crossbred fowl which was found dead, a large intra-abdominal blood clot was found proceeding from a rupture in the substance of a smoothly lobulated brownish-yellow tumour of soft consistency, measuring 6·5 × 5 × 4·3 cm. and occupying the site of the left (functional) ovary. It was freely movable, being only attached at the hilus, and was therefore easily dissected out. Numerous small cream-coloured tumours were scattered in the substance of the liver, spleen and kidneys. The adrenals seemed normal and this was subsequently confirmed by histological study.

Section of the gross specimen showed it to have a richly vascular outer zone

and an irregular inner zone, of a spongy brown character. The periphery of the growth tended to strip off in layers of what appeared to be necrotic tissue.

It is fortunate that the several blocks of tissue taken from various regions were fixed in Helly's fluid, as well as other fixatives, since it is possible that the unusual nature of the tumour would have been largely missed, had it not been chromed.

Histological examination of the outer zone shows it to be necrotic in some parts. The healthy remainder is composed of connective tissue cells with spherical to spindle-shaped nuclei, and eosinophilic granular cytoplasm, surrounding aggregations of large polyhedral cells with abundant finely granular eosinophilic cytoplasm, large nuclei and very prominent nucleoli (nuclear-nucleolar ratio 1:3 to 1:5).

An abundant infiltration with eosinophils is present in many parts of the small epithelial-cell moiety.

A well-marked but thin layer of connective tissue separates this outer zone from the inner, which is composed of small lobules of cells separated from each other by fine trabeculae of connective tissue. The cells composing these groups are large, with giant nucleoli similar to those mentioned above, and have a brownish-yellow cytoplasm in bichromate fixed haematoxylin and eosin preparations. They are frequently arranged in palisade formation at the periphery of the lobule, and their disposition and appearance in the interior of the groups is strikingly similar (save in size of nucleoli) to the cells composing the avian adrenal medulla (Fig. 15 and compare with previous figure). Areas of necrosis and spaces filled with fibrinous material are common in the inner zone, which is also very vascular.

Other regions of the tumour, and the metastatic lesions in the liver, spleen, and kidneys, consist mainly of polyhedral cells with large vacuolated nuclei and prominent nucleoli, tending to palisade small acini whose basement membrane is composed of reticulum fibrils. The cytoplasm of these cells is finely granular and heterophilic, and mitotic figures are common. At the edge of one series of sections, the only indication of ovarian origin is the presence of a few follicles. Bands of smooth muscle are scattered throughout the section.

The presence of such large numbers of cells showing a distinct chromaffin reaction justifies a diagnosis of chromaffinoma or paraganglioma. One hesitates to speak of a phaeochromocytoma of the ovary since no record of such tumour is in existence, but the term is really synonymous. The nature of the remaining cells of this tumour is debatable. Some are similar to the chromaffin cells, except there is no brown staining of the cytoplasm, and could conceivably be young forms. Others are mesenchymal in nature and are strongly reminiscent of thecal cells, a view which is supported by the fact that isolated groups of such cells are seen to be undergoing luteinization; and finally the polyhedral cells palisading the small acini which make up the rest of the tumour and metastatic growths, resemble granulosa-cells. This tumour, then, is a complex in which chromaffin, theca and granulosa-cells all occur, the latter metastasizing to the liver, spleen and kidneys.

The ovarian tumour just described apparently had no noticeable effect on the behaviour of the bird. In the human subject phaeochromacytomas are associated with raised blood pressure. It may not be entirely coincidence that this bird died as a result of an internal haemorrhage.

(6) The following case is another example of a masculinizing tumour. It occurred in a Brown Leghorn " hen " at this Centre, and was originally studied by Drs. Blyth and Carr, who supplied the following details.

The bird appeared to be a male when killed, although originally it had a normal female appearance. It was used to propagate the Duran-Reynals filterable sarcoma. *Post-mortem* examination showed a small tumour at the site of the left ovary, normal in shape for that organ, but with no macroscopically-visible ova, measuring 1·2 × 0·7 cm. It was closely adherent to the left adrenal gland, and was sectioned with it. This tumour is composed of well-developed testiculoid elements lying in a stroma of undifferentiated spindle-shaped cells (Fig. 16). Many solid cords of epithelioid cells lie just below the germinal epithelium investing the surface. The growth appears to have arisen within the adrenal capsule.

Another small (0·5 × 0·2 cm.) tumour was found in the site of the rudimentary right gonad and closely adherent to the dorsal wall. This shows not only testiculoid elements, but also a considerable area composed of whorls and strands of rapidly dividing mesenchymatous cells, presumably a metastasis from the experimentally transplanted sarcoma.

The histology of both gonadal growths resembles Ewing's (1942) Fig. 316, illustrating a " testicular adenoma of the ovary," a well-differentiated tumour belonging to the arrhenoma group. The nests of cells below the germinal epithelium resemble the primitive sex cords of the indifferent embryonic gonad. This tumour illustrates well the bisexuality of the embryonic fowl's ovary. The *rete ovarii* formed from the primitive sex cords is a homologue of the testis and as is well known can develop into one following left ovariectomy of the hen, where the right gonad hypertrophies and differentiates even to the extent of producing spermatozoa (Benoit, 1923).

Another tumour of the ovary associated with masculinization in a Light Sussex hen is shown in Fig. 17. Folliculoid formation and luteinization is apparent, and the structure is comparable to a granulosa-cell tumour. In fact, this diagnosis would be justifiable on histological grounds alone, but has to be modified in view of the androgenic effects on the host.

It would appear that the rudimentary right ovary is not the only place where in theory at any rate, testiculoid structures could arise in the intact bird. Champy, Lavedan and Marquez (1939) state that the adrenals of either male or female castrates in the fowl frequently contain " regenerating gonadal cells." Birds' adrenals constantly contain Wolffian duct remnants in the capsule on the ventral internal aspect. These slowly hypertrophy following castration to form an epididymis-like structure, whilst some form epithelial tubules similar to those found in the right ovarian rudiment subsequent to castration. Spermatogenesis has not been seen in the adrenal capsule however. There does not seem to be any record of spontaneous arrhenoma arising in the adrenal capsule of the fowl, but it seems possible that the foregoing is a case in point.

(7) The last of the tumours of the female gonad to be described was originally considered to be a teratoma. It is unfortunate that no comparison can be made of this case with the only comparable ovarian tumour of the fowl on record (Jackson, 1936), since apart from stating its didermic character, no other details were given, and the single photograph is of the gross specimen.

The present case occurred in a Brown Leghorn hen aged about 3 years, at the Poultry Research Centre. The bird had ascites and ovarian tumour was diag-

nosed. Red blood cells, macrophages and epithelial cells were seen in a smear of the centrifuged deposit from the brown turbid fluid, 140 ml. of which was withdrawn from the abdomen. After death 4 days later, an ovarian tumour was found measuring 5 × 4 × 4 cm. It was mottled pink, brownish and cream, of firm consistency and mucinous on section. Other growths, pinkish and soft, were attached to the wall of the oviduct, and to the serous investment of the duodenum, pancreas and liver.

Histologically the ovarian tumour is the only one to show complexity of structure. Immediately below a thin capsule there are nests of large pale staining epithelial cells, some of which have differentiated into cysts of varying size, lined with one or several layers of columnar epithelium. In many cases these cells are actively secreting mucus. Large areas of the tumour consist of a vascular myxo-chondromatous matrix showing all stages of development of hyaline cartilage from a myxomatous tissue to irregular islands of well developed cartilage. Complicated folded, branched and invaginated epithelial tubules, and clumps of actively dividing cells often forming folliculoid structures complete the picture (Fig. 18). The implants on the viscera show a typical folliculoid type of granulosa-cell tumour.

As with many ovarian tumours, this case is not easy to classify. At first sight two embryonic layers appear to be involved, namely mesoderm and ectoderm. The latter may be represented by the cyst epithelium forming mucinous glands, and possibly as the complex epithelial tubular structures. The remainder of the tumour is mesodermal in origin. If this is the case the presence of these derivatives of two germinal layers presumably puts it in the same category as Jackson's (1936) "didermic teratoma." Willis (1948) defines a teratoma as "a true tumour or neoplasm composed of multiple tissues of kinds foreign to the part in which it arises." The presence of cartilage can be explained by the conversion of connective tissue normally present in the ovary, to cartilage by mucoid degeneration and hyalinization. At first sight the mucus-secreting epithelial cells of the cysts seem to satisfy the definition of Willis, but such structures have been described by Harvey, Dawson and Innes (1939), in Brenner-cell tumours in the human subject, and therefore presumably arise from cells indigenous to the ovary. Also it should be noted that the same authors express the view that Brenner and granulosa-cell tumours are intimately related, an opinion also held by Willis (1948).

Is a diagnosis of teratoma justifiable ? Nicholson (1950) stresses the necessity of the identification of tissues representing all three germinal layers before a diagnosis of teratoma is made. It has been shown that in this case it is doubtful whether even two layers are in fact represented, and so the view is taken that we are here dealing with a neoplasm demonstrating the close relationship between Brenner tumour, and pseudomucinous cystadenoma, in which metaplasia of myxomatous tissue has given rise to hyaline cartilage, whilst other elements have differentiated into a mature type of epithelium secreting mucus, and an embryonic moiety represented by the granulosa-cell elements has spread by implantation to organs in the abdominal cavity.

Tumours of the Male Gonad.

1. It seems extraordinary that no report exists of spontaneous seminoma in the fowl. Champy, Lavedan and Marquez (1939) describe 4 cases of "semi-

noma " in the regenerating testes of birds following partial castration, but the identity of these growths with true seminoma seems doubtful. One of their cases exhibited metastases, but no details are given of the histology of this secondary growth or its site.

Several seminomas have been studied during the past 11 years and two of these are selected for description here. The first case was found involving the left testis of a White Wyandotte cockerel. The gland was enlarged, measuring 6·0 × 3·2 × 3·3 cm., but retained its normal shape and consistency. The right testis was normal both in size and microscopic structure, and no metastases were found.

Microscopically the left testis consists of poorly delineated lobules of pale staining epithelial cells arranged in diffuse sheets and penetrated here and there by blood vessels. Mitotic figures are frequent (Fig. 19).

The cells are mainly spherical to polygonal through mutual pressure, but the faintness of cytoplasmic outline often gives a syncytial appearance. They take on a plump spindle-shape in some areas. This tumour is the counterpart of the " embryonal carcinoma " of Chevassu (1906).

2. A second testicular tumour—also involving the left organ—was seen at autopsy in a Buff Rock cockerel. It measured 4·5 × 3·8 × 3·5 cm., and on section showed an irregular yellowish area near the caudal pole, compared with the rest of the tumour which was of a cream colour. The right gonad was atrophied.

Tissue taken so as to include the two areas showed, at subsequent histological examination, two distinct cell types. The cream-coloured area, forming about two-thirds of the tumour, is composed of typical seminoma cells, showing in many places a marked acinar structure. Luteinization of these cells is proceeding in small isolated areas. The alveoli are formed by an argyrophil reticulum, which is thickened in parts to form fibrous bands. There are numerous mitoses.

Contiguous with these cells, and in parts merging with them, are very large polygonal cells with a granular acidophilic cytoplasm and relatively small peripherally situated nuclei. These resemble the interstitial or Leydig cells of the testis and occur in the form of irregular acini separated by delicate bands of connective tissue. There are no signs of cell division in this region.

In some areas there is a gradual transition between seminoma and interstitial cells (Fig. 20), whilst others show an abrupt change separated by a zone of compressed and distorted seminomatous tissue. In this case, two distinct cell types are present as contiguous growths in the one tumour. The question arises whether the interstitial-cell moiety represents a concommitant neoplasia of the stroma, as the acceptance of Firket's (1920) view on the connective tissue origin of interstitial cells would imply. In other words, is this an example of a " mixed tumour "—or alternatively is it an indication of the teratomatous nature of seminoma, as Ewing (1942) believes, but which Willis (1948), Nicholson (1950), and others dispute ?

Still another possibility exists, namely that the two cell types have arisen from a common ancestral totipotent germinal cell capable of giving rise to a variety of tissues, in accordance with the view put forward by Innes, Harvey, and Dawson (1938).

A re-examination of Fig. 20, which is representative of most of the junction between the two components, shows that the seminoma cells and the interstitial

cells merge indistinguishably, and moreover, lie in the same connective tissue-bounded cords. Another testicular tumour in the writer's collection shows a similar transition, but this time between seminiferous epithelium and seminoma (Fig. 21), a finding which is not in accord with Ewing's (1942) hypothesis.

It therefore appears that in the fowl at any rate, seminiferous epithelium can give rise to both seminoma and interstitial-cell tumours, and that it is not necessary to invoke the theory of a one-sided development of a teratoma to explain these cases.

3. The last testicular tumour of special interest occurred in a cross-bred fowl, which showed during life a marked atrophy of the comb, and cessation of crowing and of other activities associated with the adult male bird.

At post-mortem, a greatly enlarged (9 × 10 × 6 cm.) left gonad was found, a flattened oval in shape and firm in consistency. The right testis was small, fairly rich in interstitial cells and showed little spermatogenesis upon histological examination. On medial section, the tumour presented two prominent nodules and several well-defined coloured areas varying from cream through yellow mottled with haemorrhages to reddish-brown. A prominent fleshy band ran through the centre of the growth, which also contained several cysts filled with a brownish mucinous fluid. There was a well marked vascular capsule (Fig. 22). Tissue blocks were taken from five different regions for study.

The area marked " A " in the figure, consists of small cysts or irregular glands lined with either mucus-secreting cells or flattened epithelium, lying in a stroma of mesenchymal tissue and bands of plain muscle (Fig. 23). Numerous islands of interstitial cells occur in various stages of degeneration, and frequently lying within the lumen of the glands, which otherwise contain a colloid-like material.

Area " B " is much more cellular, being composed of masses of rather loosely arranged small pale staining epithelial cells, in diffuse sheets or tending to form follicles or " rosettes." A few cysts are present, similar to those described in area " A," and one of these is partly lined with a tall columnar epithelium with basally situated nuclei and a striated free border, resembling intestinal epithelium (Fig. 24). The whole region is divided into irregular trabeculae by thin cords of plain muscle and connective tissue. It is rather vascular and in parts is heavily infiltrated with granular leucocytes. Necrotic changes are plentiful, and other parts of the tumour taken for examination show a great deal of degeneration.

The teratomatous nature of this tumour seems fairly certain, although it does not contain such a variety of tissues as the experimentally induced testicular teratomata of Bagg (1936), which incidentally, as Falin (1940), points out, offers good evidence for the theory of the multipotency of the spermatocyte. Seminomatous tissue is not present. Willis (1948) says in this connection : " I know of no example of associated seminoma and teratoma in an animal." Instead, there is in parts, epithelial tissue resembling arrhenoma, and it is an interesting though admittedly highly debatable point whether these parts are of the nature of the so-called " androma," a benign testicular tumour associated with feminization in the human subject, described by Teilum (1946), and of which only two cases have been studied. While it is doubtfully justifiable to speak of feminization in this cockerel, it did at least lose certain of its male characteristics both in appearance and behaviour. Willis (1948) mentions that certain tumours in the human ovary, designated a " combination of granulosa-cell tumour and arrheno-

blastoma " have contained lipoid-laden cells regarded as Leydig cells, and also glandular structures lined by columnar mucous epithelial cells.

DISCUSSION.

In the fowl, as in man, there exists for a brief period in embryonic development, an indifferent gonad characterized by the presence of a medullary or male component, and a cortical or ovarian part. Suppression or stimulation of this bipotential gonad during development leads to the formation of testis or ovary as the case may be. For example, it is known that the injection of oestrogen into the fertile egg at the indifferent stage (from the third to the sixth day), results in the formation of an ovo-testis in genetic males.

The phenomenon of sex-reversal is based on this bisexual organization, and it seems probable that the formation of androgen-secreting tumours of the ovary with the resulting appearance of secondary male sex characters, is due to the suppression of the female component by disease, followed by neoplastic transformation, possibly due to endocrine imbalance, of the rudimentary male component. It may be that the normal presence of a rudimentary right ovary increases the probability of this occurrence in birds.

This tendency to change to a state of " maleness " is much more pronounced than the opposite case of a generic male developing secondary female characters. The reason for this is not clear unless it is assumed that in the male embryo the cortical region of the differentiating gonad is usually completely suppressed. That this may not always be the case is shown by an instance of the development of female type feathers in a Brown Leghorn male after castration (Greenwood and Blyth, 1932). At subsequent autopsy this bird had a small nodule at the site of the right gonad, whose histological structure, in the opinion of the present writer, resembles that of a thecal-cell tumour.

Teilum's (1946) case of " androma " of the testis in man may be another example of a feminizing tumour arising from a persistent remnant of the " ovarian" cortex, and the presence of arrhenomatoid tissue in a testicular teratoma of a fowl which lost certain of its male characters has already been described in the present paper.

Turning to granulosa-cell and related tumours, the development of the ovary throws some light on the inter-relationship of these oestrogen-secreting growths. In the embryonic ovary it is commonly stated that the granulosa cells arise from coelomic epithelium derived from the genital ridge, whereas the thecal cells, being stromal in nature arise from the mesenchymal elements of the mesoderm. Examination of the early ovary, however, shows primary follicles composed of a central ovocyte surrounded by a ring of indifferent epithelial cells indistinguishable from the stromal cells composing the rest of the organ. These indifferent cells appear to be the precursors of the granulosa cells, whereas the remainder of the stroma forms the theca. It seems probable that this common ancestry is reflected in the behaviour of thecal and granulosal cells in the neoplastic state, where both secrete oestrogens as judged by the muscular and glandular hypertrophy of the oviduct of the tumour-bearing bird, and both undergo the phenomenon of luteinization.

Granulosa-cell tumours in the fowl are not morphologically identical to these in man in that they do not exhibit the characteristic " rosettes " which are often

a feature of human tumours, and which are considered to be identical to the bodies of Call-Exner occurring normally in the stratum granulosum of the human ovary. Such bodies are not found in the granulosa layer of the ovarian follicle because this is usually only one, or at the most two or three cells thick. Call-Exner bodies are usually considered to be due to cystic degeneration of granulosa cells. This does not occur in avian tumours where there is ample space for such a change to take place, and it therefore seems possible that these structures may serve some specific purpose as for example a glandular function, in the ovaries of those animals in which they occur.

With regard to thecal-luteal cells and the interstitial cells of the testis, it is interesting to note that they appear to have a common ancestory. Fell (1924) states that the ovarian thecal-luteal cells of the fowl are derived from remnants of aborted medulla in the development of the ovary. Thus they have the same histogenesis as seminiferous tissue. We have already seen that the testicular interstitial cells appear to be derived from seminiferous epithelium. This common embryonic origin may account for the confusion regarding the so-called

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EXPLANATION OF PLATES.

FIG. 1.—Brenner tumour, showing cysts in various stages of development, with epithelium varying from flattened to a columnar mucus-secreting type, lying in hyperplastic ovarian stroma. H. & E. × 85.

FIG. 2.—Section from the same tumour showing nests of epithelioid cells, one of which is being transformed into a cyst. The stroma is myxomatous in this region. H. & E. × 370.

FIG. 3.—Hilar region of ovary of fowl, showing the parovarium. H. & E. × 135.

FIG. 4.—Granulosa-cell tumour of ovary. × ⅓.

FIG. 5.—Granulosa-cell tumour showing cylindroid type with a trabecular-like stroma. H. & E. × 190.

FIG. 6.—Granulosa-cell tumour showing folliculoid type, developing from solid masses of cells. The stroma is hyalinized. H. & E. × 320.

FIG. 7.—Section of a liver implant from a granulosa-cell tumour, showing areas of luteinization. H. & E. × 320.

FIG. 8.—Thecal-cell tumour of ovary. × ⅔.

FIG. 9.—Structure of the above, showing vacuolated cells, due to removal of lipoids, to the left (some nuclei are pyknotic) and cells resembling fibrous tissue to the right of the figure. H. & E. × 400.

FIG. 10.—Transection of an ovarian fibroma weighing 570 g. Note the small regressing ovary.

FIG. 11.—Adrenal cortical ? tumour of ovary. H. & E. × 380.

FIG. 12.—Group of thecal luteal cells from an active ovary. Masson's stain. × 515.

FIG. 13.—Corpus luteum derived from granulosa cells. Normal active ovary. H. & E. × 370.

FIG. 14.—Adrenal cortical and medullary cells. Bichromate fixed. H. & E. × 400.

FIG. 15.—" Chromaffinoma " of ovary. Note conspicuous nucleoli and attempted orientation of cells. Bichromate fixed. H. & E. × 370.

FIG. 16.—Testiculoid tubules in " ovary." A well differentiated arrhenoma which appeared to have arisen in the adrenal capsule. H. & E. × 370.

FIG. 17.—Folliculoid formation and luteinization in an arrhenoma. Note resemblance to granulosa-cell tumour. H. & E. × 330.

FIG. 18.—" Mixed ? " ovarian tumour, consisting of areas of tortuous glands, mucinous cysts, cartilage, solid nests of epithelial cells and a myxomatous stroma. H. & E. × 42.

FIG. 19.—Seminoma or " embryonal carcinoma " of the testis. H. & E. × 370.

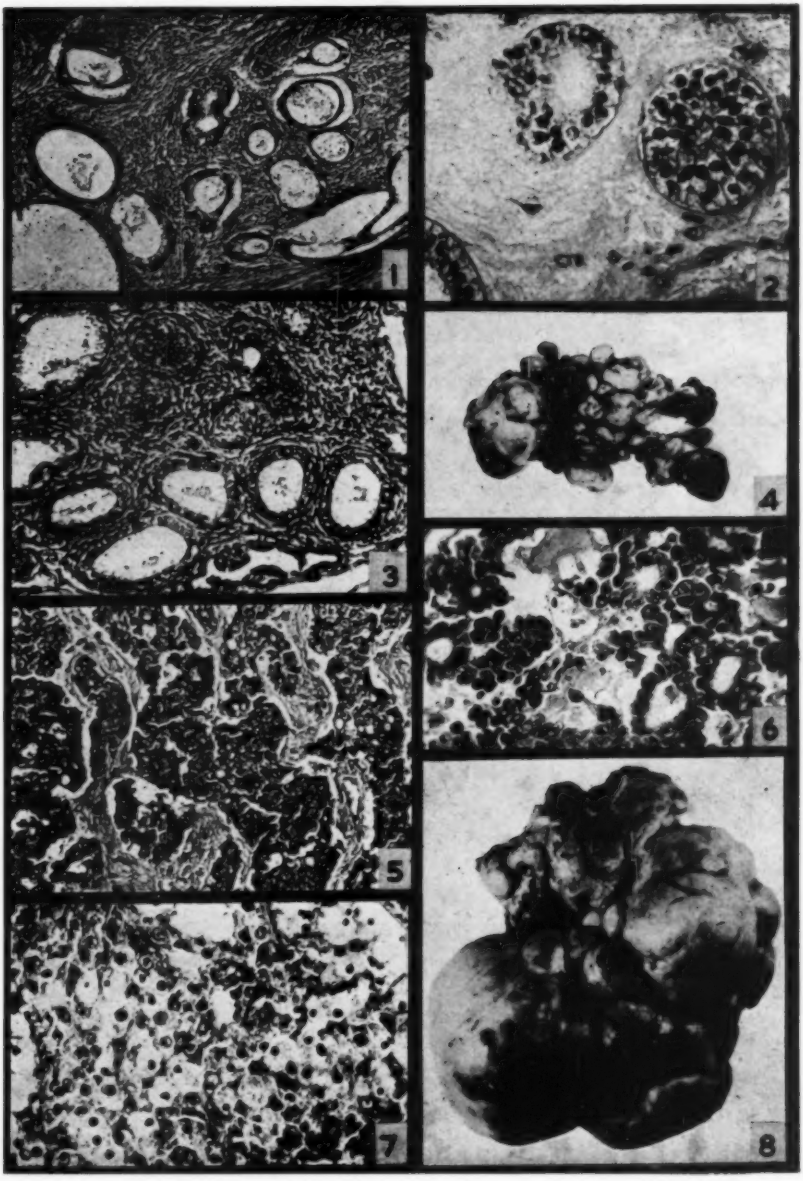
FIG. 20.—Transition zone between interstitial cell tumour (top left) and seminoma. Note that the cells lie within common connective tissue-bounded columns. H. & E. × 370.

FIG. 21.—Section showing seminoma cells differentiating into primary spermatocytes. H. & E. × 370.

FIG. 22.—Teratoma of testis, showing the two main nodules, cysts, a central muscular band, and well formed capsule. × ⅔.

FIG. 23.—Section from nodule " A " (Fig. 22), showing epithelial cysts, connective tissue and plain muscle. H. & E. × 85.

FIG. 24.—Section from nodule " B " (Fig. 22) showing tendency of cells to form folliculoid structures, and part of a cyst lined with intestinal epithelium. H. & E. × 370.



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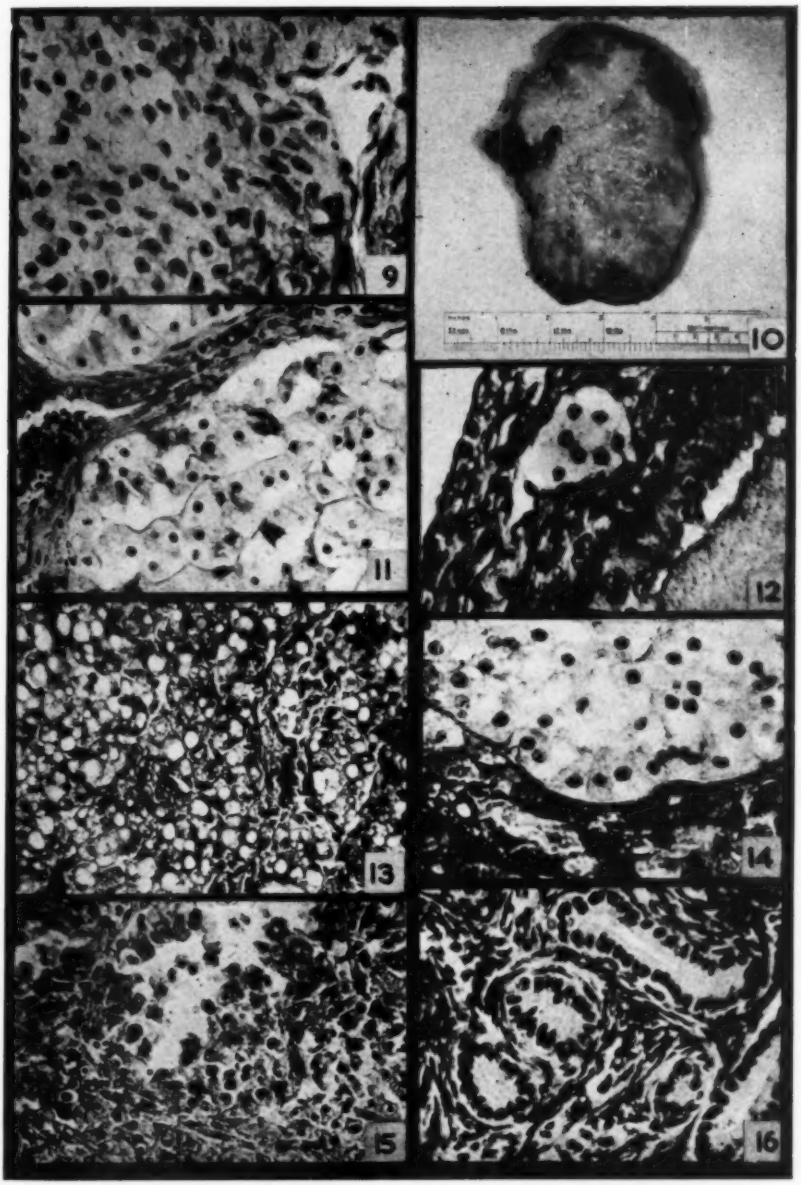

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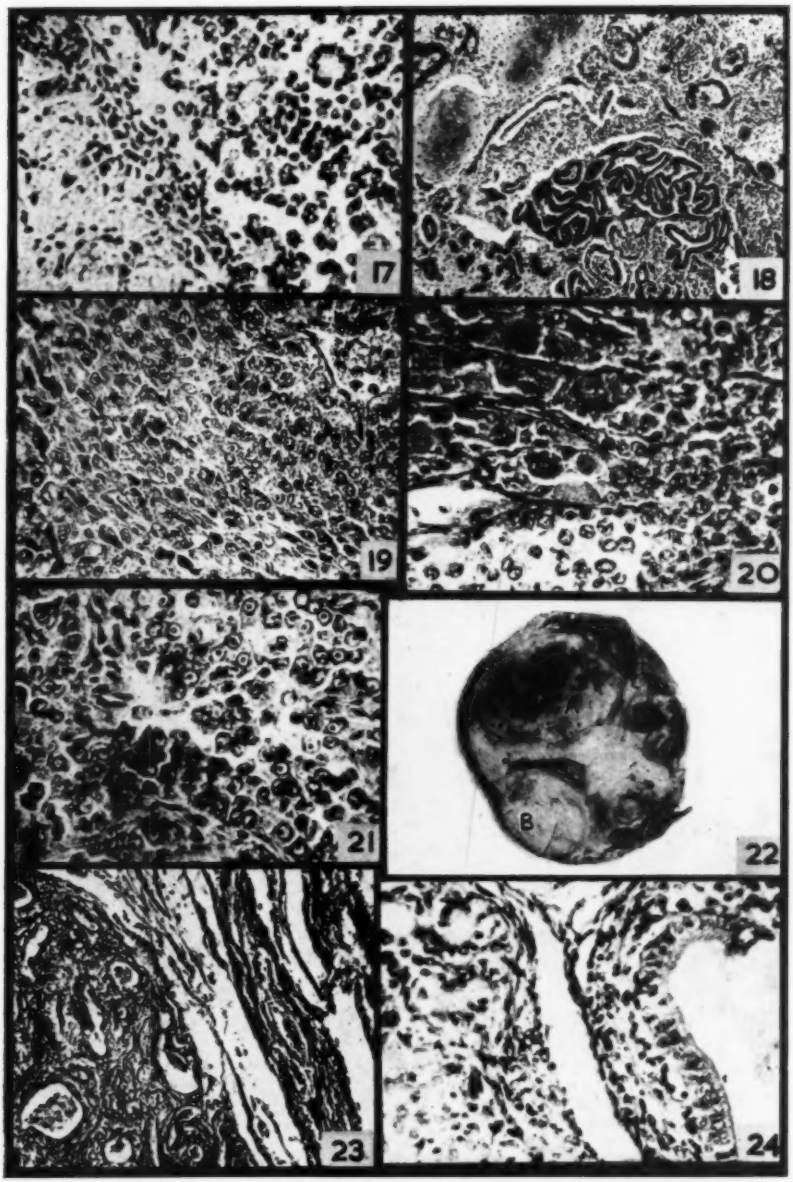

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" luteal " cells of the testis which were originally recorded by Boring and Morgan (1918), and regarding the nature of which there has been much debate.

If it is the case that cells in the testis may occasionally resemble luteal cells and exert an oestrogenic effect on the plumage of the male bird, then tumours of such cells, perhaps in a more anaplastic state, but still capable of elaborating oestrogen would account for the so-called " androma " of Teilum (1946), which is apparently indistinguishable from an arrhenoma.

Conversely, ovarian growths resembling luteal-cell tumours may secrete androgens, e.g., arrhenomas, regarding which Burrows (1943) states (in the human subject), " Some ovarian tumours which induce hirsuties and other masculine phenomena are the colour of corpora lutea, and are composed of cells which resemble those of luteal tissue. Others might be described from their cytological appearance as thecomas, or as granulosa-cell tumours, and yet others look like tumours derived from adrenal tissue."

It should not be forgotten that arrhenomas and seminomas may also undergo luteinization. In view of the origin of all these tumours in tissues with similar embryonic derivation, of their capacity for secreting either male or female sex-hormones independent of their cytology, and of the fact that luteinization is common to all, it seems reasonable to conclude that there is a very close relationship between ovarian tumours and between these and tumours of the testicle. This relationship tends to be obscured by the present confusing system of classification, which could well be revised.

In conclusion it is considered that the suggestion of Burrows (1943), should be more widely adopted, i.e., that tumours secreting androgens should be called arrhenomas, and tumours secreting oestrogens should be called theelomas, irrespective of their histological structure or site of origin in the body. Only by adopting some such simple and logical classification (with additional details where necessary regarding malignancy, etc.) will order be brought to the unwieldy and unscientific classification of gonadal tumours, adopted by most present-day pathologists.

SUMMARY.

1. Most, if not all, of the rarer ovarian and testicular tumours of the human subject have their counterparts in the fowl.

2. Brenner tumours appear to arise from the parovarium and not from Walthard nests. They show affinities to granulosa-cell tumours and to pseudo-mucinous cystadenomata.

3. Granulosa and theca-cell tumours are characterized by their oestrogenic effect as evidenced by a hypertrophied oviduct in the non-laying fowl. Both types undergo luteinization. Call-Exner bodies are not present in the thin granulosa of the fowl ovary, and typical rosettes were not found in granulosa-cell tumours.

4. The occurrence of both adrenal cortical and chromaffin tumours in the ovary is adduced as evidence in favour of the occasional presence of heterotopic adrenal tissue in that organ, presumably due to developmental displacement.

5. Arrhenomata may not only arise from the rudimentary right ovary, but also from Wolffian duct remnants within the adrenal capsules. These essentially masculinizing tumours may also undergo luteinization.

6. The phenomenon of luteinization is also to be seen in seminomas.

7. Seminomas arise from seminiferous epithelium and can give rise to cells indistinguishable from Leydig's cells.

8. A testicular teratoma showed regions indistinguishable from arrhenoma. The host had lost much of its male characteristics.

9. It is evident that there is a very close relationship between the various ovarian tumours, and between these and tumours of the testicle.

REFERENCES.

Bagg, H. J.—(1936) *Amer. J. Cancer*, **26**, 69.
Benoit, J.—(1923) *C.R. Soc. Biol. (Strasbourg)*, **89**, 1326.
Boring, A. M., and Morgan, T. H.—(1918) *J. gen. Physiol.*, **1**, 127.
Burrows, H.—(1943) *J. Obstet. Gynaec.*, **50**, 430.
Campbell, J. G.—(1945) *J. comp. Path.*, **55**, 308.
Champy, C., Lavedan, J. P., and Marquez, J.—(1939) *Arch. Zool. exp. gén.*, **80**, 389.
Chevassu, M.—(1906) 'Tumeurs de testicule.' Paris. (Monograph.)
Ewing, J.—(1942) 'Neoplastic Diseases.' London (W. B. Saunders Co.).
Falin, L. I.—(1940) *Amer. J. Cancer*, **38**, 199.
Fell, H. B.—(1924) *Brit. J. exp. Biol.*, **1**, 293.
Firket, J.—(1920) *Arch. Biol., Paris*, **30**, 393.
Friedgood, H. B., and Uotila, U. U.—(1941) *Endocrinology*, **29**, 47.
Glynn, E. E.—(1921) *J. Obstet. Gynaec.*, **28**, 23.
Greenwood, A. W., and Blyth. J. S. S.—(1932) *J. Genet.*, **26**, No. 2, 199.
Harvey, W. F., Dawson, E. K., and Innes, J. R. M.—(1939) *Edinb. med. J.*, **46**, 256.
Innes, J. R. M., Harvey, W. F., and Dawson, E. K.—(1938) *Ibid.*, **45**, 36.
Jackson, C.—(1936) *Onderstepoort. J. Vet. Sci.*, **6**, No. 1 (Monograph).
Maximow, A. A., and Bloom, W.—(1948) 'Textbook of Histology.' London (W. B. Saunders Co.).
Meyer, R.—(1931) *Amer. J. Obstet. Gynec.*, **22**, 697.
Nicholson, G. W. de P.—(1950) 'Studies on Tumour Formation.' London (Butterworth & Co.).
Olson, C., and Bullis, K. L.—(1942) *Mass. Agric. exp. Sta. Bull.*, 391.
Seifried, O.—(1923) *Z. Krebsforsch.*, **20**, 188.
Sheather, A. L.—(1911) *J. comp. Path.*, **24**, 129.
Teilum, G. (1946) *Acta path. microbiol. scand.*, **23**, 252.
Walthard, M.—(1903) *Z. Geburtsh. Gynäk*, **49**, 233.
Willis, R. A.—(1948) 'Pathology of Tumours.' London (Butterworth & Co.).

THE PREPARATION AND ASSAY OF THE ROUS No. 1 SARCOMA AGENT.

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THE major requirement for the investigation of the physical and chemical properties of the Rous No. 1 sarcoma agent is a source of " purified " agent of a uniformly high infectivity, and which may be stored without loss of this activity, since it is not always convenient to compress the investigation to within the relatively short period in which the purified agent is stable *in vitro*. This paper describes a method for the production of concentrates of the virus in a form suitable for storage, and the storage problem itself is considered in the following paper.

A variety of chemical and physical methods have hitherto been used for separating and concentrating the agent from biologically-inactive tumour protein and nucleoproteins.

Claude (1935*a*) found that such contaminants could be removed by adsorption on to freshly prepared aluminium hydroxide gel, and the carbohydrate contaminant by precipitation with gelatin. The agent was not directly affected, and a combined chemical fractionation and dialysis gave a 25-fold concentration in terms of dry weight (Claude, 1935*b*).

This method was also investigated by Dmochowski (1948*b*), who recorded a 50 per cent increase in the activity of the agent compared with the starting filtrate.

Shemin, Sproul and Jobling (1940), and Shemin and Sproul (1942) precipitated the agent from filtrates by addition of papain or of histone. The papain-agent complex (containing 50 to 70 per cent papain) was found to be at least as active as the original filtrate. The papain could be removed by fractional centrifugation after dissociation of the complex in salt solution. Amounts of agent corresponding to as little as 7×10^{-7} mg. nitrogen (the range was $1{\cdot}3 \times 10^{-3}$ mg. to 7×10^{-7} mg.) were found to be infective.

Concentration of the agent by centrifugation was first described by Ledingham and Gye in 1935 and fractional centrifugation methods have since been widely used (McIntosh, 1935; Amies, 1937; Claude, 1937, 1938, 1939, 1940; Claude and Rothen, 1940; Pollard, 1938, 1939; Stern and Duran-Reynals, 1939; Dmochowski, 1948*a*). Claude (1938, 1939) isolated a fraction corresponding to less than 3·5 per cent of the dry weight of the original extract which, in amounts corresponding to $4{\cdot}0 \times 10^{-10}$ mg. N, produced tumours in 13 to 17 days after subcutaneous inoculation into fowls. There are two difficulties in the separation and concentration of the agent by fractional centrifugation: (*a*) the viscosity of the initial tumour extract—which contains a mucoprotein, and (*b*) the tendency of the agent to aggregate, and to adsorb to tissue components.

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Pollard (1939) and Amies and Carr (1939) were able to overcome the first difficulty by precipitating the agent from a clarified extract by adjusting the pH to 5·0 to 5·5. The isoelectric point of the purified agent is 3·5, but tumour extracts deposit a mucoprotein at pH 5·0, which takes down the agent with it. The aggregated agent, etc., was then further concentrated by a low-speed centrifugation procedure. The aggregates were broken down by tryptic digestion at pH 8·8 to 9·0 and the released agent further "purified" by fractional centrifugation.

Alternatively, the mucoprotein may be hydrolysed by incubation of the extract with hyaluronidase, which has no deleterious effect upon the agent (Sturm and Duran-Reynals, 1932; Shemin and Sproul, 1942). The agent may then be deposited directly from the non-viscous extract.

Bryan, Riley, Deihl and Voorhees (1947) have avoided this difficulty by the simple, but uneconomical, procedure of rejecting the first, viscous extract.

A further method which has been claimed to apply to the purification of small amounts of agent is that of chromatography on Celite (Riley, 1948). The agent is strongly adsorbed to Celite from physiological salt solution, but may be eluted with very dilute (0·001 M) salt solution. In terms of the ratio, biological activity : nitrogen content, a 3- to 7-fold enrichment was obtained from a partially-purified tumour extract. Three of the above methods, papain precipitation, fractional centrifugation after precipitation at pH 5·0 and direct deposition after treatment of the initial extract with hyaluronidase, have been reinvestigated from the point of view of the preparation of large quantities of agent in a form suitable for storage. A further method, methanol precipitation, hitherto applied to a number of other animal viruses, has also been investigated.

Cox, van der Scheer, Aiston and Bohnel (1947) found that influenza virus (from allantoic fluid) could be precipitated in active form from suspensions in phosphate buffer at pH 7·0 to 8·0 by addition of methanol to a final concentration of 15 to 30 per cent. The method was applied to psittacosis virus purification by Wagner, Golub and Andrew (1948) and to mouse poliomyelitis by Brunfield, Stulberg and Halvorson (1948). Fischer (1949) found, however, that rabbit papilloma virus was reduced in titre after such precipitation and Moyer, Sharpless, Davies, Winfield and Cox (1950) have recently stressed the necessity for using 0·3 M buffer solutions for the extraction of influenza virus from the methanol precipitates.

MATERIALS AND METHODS.

Tumour.

The Rous No. 1 tumour used was grown throughout in 6-week-old Brown Leghorn fowls from Dr. Greenwood's Edinburgh flock. Only fast-growing tumours, 20 to 30 days old were used. Such tumours are known to contain similar amounts of virus with little or no associated inhibitory material.

1 : 10,000 HCN.—One ml. M KCN was added to 8 ml. water and neutralized to phenol red with N HCl; 1 ml. added to 1 l. buffer gives a final concentration of 1:10,000.

Buffers.—In the pH range 2·2 to 9·0, McIlvaine's (1921) phosphate-citric acid buffer has been used. The buffer components are 0·2 M Na_2HPO_4 and 0·1 M citric acid. Of each buffer 50 ml. was diluted before use to 1 l. (0·005 M buffer).

Lemco broth.—Used as supplied by Baird & Tatlock.

Trypsin.—Crystalline trypsin (Armour Laboratories) containing 50 per cent magnesium sulphate.

Hyaluronidase.—Fresh rat testes were homogenized (Waring " blendor ") with 10 vol. water. The suspension was roughly filtered through surgical gauze and frozen-dried in 10 ml. aliquots in 25 ml. McCartney bottles. The contents of each bottle were reconstituted before use by the addition of 10 ml. water.

Papain.—British Drug Houses, Ltd.

Centrifuges.

The Rous agent becomes progressively more thermolabile as purification proceeds. Centrifuge B was used in a cold-room at 0° C., and C was adjusted to a chamber temperature of 0° C. The Sharples centrifuge (A) was cooled with running tap water only. The centrifugal procedures used are listed below and will be referred to in the rest of the paper by such abbreviations as (A, i).

A. Sharples air-driven supercentrifuge, stainless steel bowls.—(i) Clarification; clarifier bowl; maximum flow rate at 25,000 r.p.m. (ii) Deposition at pH 5·0: Same bowl and conditions as (i). (iii) Deposition at pH 7·0. The same bowl as (i) and (ii), but 30 ml./minute flow at 48,000 r.p.m.

B. Serval angle centrifuge, Type SS.1, lusteroid tubes.—(i) Clarification; 5000 r.p.m. (3000 g.) for 20 minutes. (ii) Deposition at pH 7·0; 11,000 r.p.m. (14,400 g.) for 55 minutes.

C. International refrigerated centrifuge, Type PR-1.—High speed attachment and angle head No. 296; lusteroid tubes. (i) Clarification; 5000 r.p.m. (2000 g.) for 18 minutes. (ii) Deposition; 15,000 r.p.m. (17,000 g.) for 57 minutes.

Waring blendor efficiency.

Doubt has frequently been expressed, usually without supporting evidence, concerning the use of the Waring " blendor " for the maceration of tissues for the liberation of enzymes and viruses. It would obviously be tiresome to " mince " half-kilogramme batches of tumour with the " conventional " scissors and accordingly the efficiency of the Waring " blendor " was tested in the following way.

Tumours from two fowls were removed and small samples, totalling about 5 g., taken. The bulk of the tumour was extracted in the Waring " blendor " with 10 volumes of water containing 1:10,000 HCN, and the small sample " minced with scissors " and extracted into a similar medium. Suitable dilutions of these extracts were injected into fowls. The Waring " blendor " extract gave tumours at a dilution corresponding to 10^{-5} g. of tumour, while the other failed to give tumours at an equivalent of 10^{-4} g.

I. Precipitation with Papain.

In the Waring " blendor " 44 g. fresh tumour tissue was disintegrated with 440 ml. 0·005 M buffer at pH 7·2 containing hyaluronidase and 1:10,000 HCN. The tissue suspension was clarified (B, i) and the agent deposited from the supernatant (B, ii). The combined pellets were resuspended in 25 ml. water by gentle pipetting and the suspension clarified (B, i). Aliquots of 1 ml. of the supernate were added to (*a*) 5 ml. water (control), (*b*) 5 ml. papain solution (Shemin and Jobling, 1940; Shemin and Sproul, 1942) and (*c*) 5 ml. 0·1 per cent trypsin (B.D.H.). The mixtures were then incubated at 37° C. for 1 hour. Tube (*b*)

developed a precipitate which was deposited. The control suspension (*a*) was assayed comparatively against (*b*)—resuspended papain-agent complex, etc., (*b*) —supernatant and (*c*). The assay showed that (*b*) deposit and (*c*) were devoid of any activity, (*b*) supernatant was weakly active and (*a*) control was highly active at $1{\cdot}0 \times 10^{-3}$. In view of the results obtained by Pirie in 1933, the inactivation by crude commercial trypsin was not unexpected, but the result with (*b*) was.

II. Precipitation with Methanol.

(1) A fresh tumour tissue suspension was prepared by disintegration in the Waring " blendor " with 10 vol. of water containing 1:10,000 HCN. This suspension was clarified (A, i). The supernatant was then cooled to — 12° C. and methanolic buffer solutions prepared, and cooled to — 5° C. These buffers contained (*a*) 40 per cent methanol, (*b*) 50 per cent methanol and (*c*) 60 per cent methanol respectively in 0·005 M buffer. Equal volumes of (*a*), (*b*), (*c*) or buffer (*d*)-control, were added to volumes of the supernatant. The four groups were stored at 0° to 2° C. for 2 hours and then clarified (B, i). The supernatants (Sa, etc.) were set aside for assay and the deposits resuspended in an equal volume of 0·005 M buffer and re-clarified. These supernatants (Saa, etc.) were also assayed. Virus activity was retained in Sd, Sa and Sc. Activity was also found in Saa and, doubtfully, in Sbb ; Scc had no activity.

It was concluded that the precipitation with methanol was incomplete even at a concentration of 30 per cent and that methanol concentrations greater than 25 per cent tended to attenuate any precipitated agent.

(2) Method 1 was followed for the preparation of the initial clarified tumour extract. The control virus suspension was prepared by deposition from the clarified extract at pH 5·0 (A, ii), followed by resuspension of the deposit at pH 7·0. An equal volume of 50 per cent methanolic buffer was added under the same conditions as Method 1 and the resuspended deposit assayed against the extract. The assay showed that the alcohol-deposited agent had only 1 per cent of the activity of the control suspension.

(3) A similar clarified extract was prepared, cooled to 0° C. and the conditions of Method 2 again followed. Larger volumes were used and the alcohol-precipitated agent was deposited at low speed (A, i). The deposit was resuspended in 0·005 M buffer at pH 7·2, clarified (B, i) and the agent in the supernatant deposited (B, ii). The pellet was finally resuspended in 0·005 M buffer, pH 7·2, adjusted in concentration to the equivalent of 1 g. tumour tissue/ml. (10°—see Biological Assay) and assayed against the extract, similarly adjusted. The control contained $>10{,}000$ minimal infective doses per ml., whereas the alcohol deposited agent contained <1000 m.i.d. per ml.

III. Deposition after Precipitation at pH 5·0.

A 10 per cent fresh tumour tissue suspension was prepared in the usual way, but with the omission of hyaluronidase. The suspension was clarified (A, i) and deposited (A, ii) after adjustment to pH 5·0 by addition of an equal volume of 0·005 M buffer at pH 5·0.

Fractional centrifugation.

The deposit from (A, ii) was resuspended in 0·005 M buffer at pH 7·0 (v/w of original tumour tissue taken) and clarified (B, i). The supernatant, S (Fig. 1)

was decanted and the deposit D, re-extracted with a similar volume of the same buffer and re-clarified (B, i) giving a deposit D_3 and a supernatant, S_3.

The agent, etc., of supernatant S was deposited (B, ii). The supernatant (S_1) was rejected and the agent deposit (D_1) re-extracted with 0·005 M buffer at pH 5·0 (25 per cent v/w original weight tumour) and re-clarified (B, i). This supernatant (S_2) was adjusted to pH 7·0, and deposit (D_2) resuspended in the same volume of 0·005 M buffer at pH 7·0. D_2, S_2 and S_3 were assayed in the usual way. D_2 contained more than 10,000 m.i.d./ml.; S_3, not more than 1000 and S_2 not more than 100 m.i.d./ml. (Fig. 1).

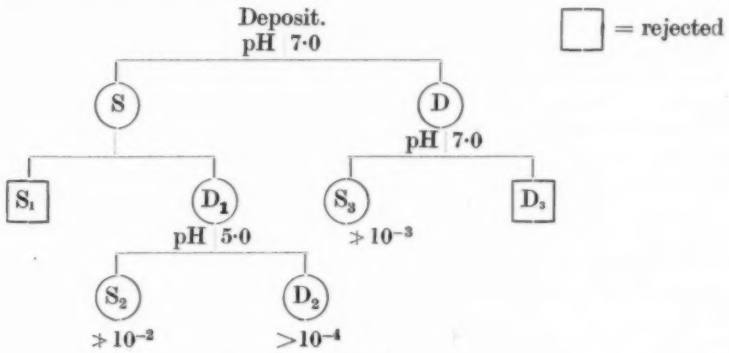

FIG. 1.

These results show that this method, although used successfully by Pollard (1939) and by Amies and Carr (1939), had certain limitations. With an initial volume of tumour extract of 5 litres (from 500 g. tumour) the volumes of suspension to be centrifuged at the pH 5 deposition stage were very large. One of the major difficulties with the Rous agent is its tendency to aggregate and procedures which attempt to isolate it by aggregation ought, therefore, to be avoided. The loss by aggregation is well illustrated in Fig. 1, since $S_3 \triangleq D_2$ in titre (where S_3 derives from the second extract of the material aggregated at pH 5·0 for deposition). Moreover, deposit D_1 lost agent after *extraction* with 0·005 M buffer at pH 5·0 (S_2 assayed at $\Rightarrow 10^{-2}$) and the efficiency of the initial deposition process was thereby called into question.

The main reason why direct deposition of the agent from a clarified extract has not hitherto been attempted *on a large scale* is simply that suitable centrifuges have not been available. The air-driven Sharples supercentrifuge with a maximum speed of rotation of 50,000 r.p.m. (62,000 g.) was used by Stanley in 1945 for the concentration of influenza virus for vaccine production. Under such conditions the Rous agent may be deposited from a continuously-flowing non-viscous (i.e. hyaluronidase-treated) extract.

IV. Direct Deposition with the Sharples Centrifuge.

1. *Boundary phenomenon.*

In the Waring " blendor " 145 g. fresh tumour tissue was disintegrated with 10 volumes of 0·005 M phosphate buffer at pH 7·2 containing hyaluronidase and

1:10,000 HCN. The suspension was clarified (A, i) and the virus deposited from the supernatant (A, iii). For deposition of the virus the stainless- steel centrifuge bowl was invariably lined with cellophane (7/1000 in.) which may (*a*) be readily withdrawn and which (*b*) minimizes contact of the virus with metals.

When unrolled this " liner " invariably showed a boundary, the distance of which from the bottom of the bowl, varied with the rate of flow of the virus suspension through the centrifuge. In terms of total nitrogen distribution, the deposit was uniformly distributed above and below the boundary, although the virus content of the top and bottom fractions differed considerably. The " liner " was therefore divided at the boundary and the deposits removed and separately resuspended in 0·01 M buffer at pH 7·2 (D_B and D_T). A few mg. per cent crystalline trypsin was added to each, the suspensions incubated at room temperature for 40 minutes and the virus re-deposited (A, iii) (S_B, S_T-supernatants and D_{B_1}, D_{T_1}-deposits). The separate fractions were analysed for total nitrogen. In this second deposition procedure neither fraction gave a boundary indicating that the material below the initial boundary was largely trypsin-digestible.

The results may be tabulated.

TABLE I.

Fraction.	N, mg.	N, per cent total N.	Comparative assay.
Bottom.			
D_B	49·5	51·5	—
S_B	43·2	45·0	—
D_{B_1}	6·3	6·5	10^{-2}
Top.			
D_T	47·0	48·5	—
S_T	21·4	22·2	—
D_{T_1}	25·5	26·4	10^{-3}

Trypsin digestion removed 87·5 per cent of the nitrogen of the bottom fraction, but only 45·5 per cent of that of the top fraction. Overall 67 per cent of the total nitrogen was thus removed. The virus concentrates ($D_{B_1} + D_{T_1}$) contained 31·8 mg. nitrogen (33 per cent of the total nitrogen) of which 80 per cent (25·5 mg.) was recovered from the top fraction. The fractions D_{B_1} and D_{T_1} were assayed comparatively at 10^{-1}, 10^{-2} and 10^{-3}, and the figures recorded do not refer to the m.i.d.

2. *Nitrogen distribution after trypsin treatment.*

In the Waring " blendor " 520 g. fresh tumour tissue was disintegrated with 10 volumes of 0·005 M phosphate buffer at pH 7·2 containing 1:10,000 HCN—but without hyaluronidase—and the suspension clarified (A, i). Four litres of extract was obtained which was then treated with clarified (C, i) rat testes extract and to 2 l. of which was added *c.* 0·5 mg. per cent crystalline trypsin. The non-trypsinized half (X) was deposited (A, iii), the " liner " removed and divided at the boundary. The top (XT) fraction was resuspended in 0·005 M buffer, pH 7·2 and clarified (B, i). The supernatant from this (XTS) was assayed in the usual way.

The trypsinized half (Y) was first re-clarified (A, i) to remove a slight deposit which had formed, and the agent then deposited (A, iii). No boundary was found on the " liner " and the total deposit (YD) was resuspended in buffer and clarified (B, i). The supernatant (YDS) was assayed. Fractions were analysed for total nitrogen (Table II).

TABLE II.

Fraction.	N, mg.	N, per cent total N.
X (no trypsin)	1310	100
Total deposit on liner	208	16
XTS	13·8	1·05
Y (trypsin)	1310	100
Total deposit on liner	81·5	6·2
YDS	22·2	1·7

The virus-containing fractions, XTS and YDS, contained a total of 36 mg. nitrogen (1·375 per cent total nitrogen in original clarified tumour extract).

Assays showed that the trypsin-treated fraction (YDS) not only contained more nitrogen than the non-trypsinized XTS, but that, at a dilution of 10^{-6}, YDS was more active than XTS. The trypsin removed again some 61 per cent of the nitrogen of the treated fraction.

3. *Efficiency of fractional centrifugation.*

A " liner " deposit was prepared from 110 g. fresh tumour tissue in the usual manner (p. 88).

The deposit (D_1) was resuspended in 100 ml. 0·005 M buffer, pH 7·0, and treated with crystalline trypsin for 1 hour at room temperature. (*a*) The suspension was then clarified (B, i), the deposit (D_2) discarded and (*b*) the virus deposited (B, ii) from the decanted supernatant (S_1). The supernatant (S_2) from this deposition was discarded and the deposit (D_3) resuspended in 60 ml. buffer and (*c*) re-clarified (C, i). This deposit (D_4) was rejected and virus again (*d*) deposited (C, ii) from the supernatant (S_3). The supernatant (S_4) from this deposition was assayed. The deposit (D_5) was resuspended in 30 ml. buffer and (*e*) re-clarified (C, i). The deposit (D_6) was negligible. The supernatant (S_5) was assayed in the usual way.

Results :

S_5 contained more than 10^7 m.i.d. per ml.
S_4 contained not more than 10 m.i.d. per ml.
(Tumours in 2 out of 4 fowls at dilution 10^{-1}.)

The separation is shown schematically in Fig. 2.

The results show that, although virus losses may be expected to occur by aggregation and removal in clarification procedures (into deposits such as D_2 and D_4) no loss occurs in the deposition stages, such as (*d*), since the amount of residual virus in S_4 was infinitesimal.

4. *Standard procedure.*

This procedure has been adopted for routine preparation of a virus concentrate suitable for preservation by freeze-drying.

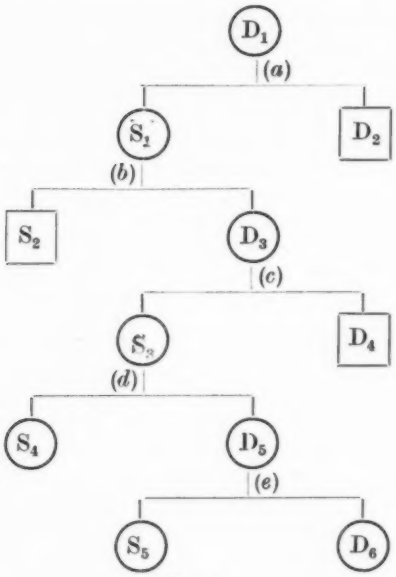

FIG. 2.

A " liner " deposit was prepared from 375 g. fresh tumour tissue by the method described on p. 88.

The " liner " was removed and the total deposit (D_1) resuspended in 375 ml. 0·005 M phosphate buffer at pH 7·5 to 8·0 containing a few mg. per cent crystalline trypsin. The suspension was incubated at room temperature for 1 hour, clarified (B, i) giving a deposit D_2 (rejected) and a supernatant (S_2) from which the virus was deposited (B, iii). The supernatant (S_3) from this deposition was rejected and the deposit (D_3) resuspended in 300 ml. Lemco broth, distributed into McCartney bottles in 10 ml. aliquots and frozen-dried. Each fraction was assayed for protein nitrogen (insoluble in cold, 5 per cent trichloracetic acid) and total nitrogen.

The results are given in Table III.

TABLE III.

Fraction.	Protein N, mg.	Non-protein N, mg.	Protein N as per cent total N.
Clarified tumour extract	1760	464	79
D_1	308	14	14
D_1 (trypsinized)	260	62	11·7
S_2	184	62	8·25
D_2	76	—	3·4
S_3	140	62	6·3
D_3	44	—	2·0

The results show that 44 mg. of the 308 mg. protein-nitrogen of the original Sharples deposit appeared as "virus concentrate" (D_3). Of the remaining 264 mg., 48 mg. appeared as acid-soluble nitrogen after trypsinization, 76 mg. was discarded as aggregated material (D_2) and 140 mg. was not sedimented under conditions in which the virus was deposited (i.e. this material was partially degraded by the trypsin). In this experiment 188 mg. N out of 308 mg. was removed as a result of the enzyme treatment. This amount (61 per cent) is in good agreement with the results of IV, 1 (67 per cent) and IV, 2 (61 per cent).

DISCUSSION.

The results of the first three methods investigated show that in our hands neither papain precipitation nor methanol precipitation gave encouraging evidence of the concentration of active agent. Deposition by pH adjustment to pH 5·0 gave material with a high titre but had two major disadvantages in that large volumes of suspension had to be handled, and the agent, once aggregated, was very difficult to redisperse.

Investigation of the direct deposition of the agent by high-speed continuous flow centrifugation showed that the bulk of the heavy protein-containing material which sedimented more rapidly could be hydrolysed by short treatment with purified (crystalline) trypsin at room temperature at pH 8·0 to 9·0. Over 60 per cent of the nitrogen of the total deposit was rendered non-sedimentable by this treatment and the activity of the agent was apparently unimpaired. Virus "concentrates" thus prepared contained some 1·7 to 2·0 per cent of the nitrogen of the original extract and had titres greater than 1,000,000 m.i.d. per ml./g. tumour.

Such comparatively "unpurified" material as YDS (Table II) had an m.i.d. of less than 1×10^{-8} mg. N—ten times as good, in fact, as the material obtained by Shemin and Jobling (1940) by papain precipitation.

It is difficult to express the "purity" of a virus preparation except in terms of a large number of factors, but the "concentrates" obtained by the above methods have been freed of more than 98 per cent of non-virus nitrogen, may readily be stored (Carr and Harris, 1951), recovered and subjected to further procedures designed to increase the physical, chemical and biological homogeneity of the agent.

The physical and chemical behaviour of the agent will form the substance of a later communication.

Biological assay.

The various methods which have been used for the determination of the tumour-producing activity of Rous agent preparations fall essentially into two groups. In the first group serial dilutions are made and injected into fowls. The highest dilution at which a tumour is produced gives a "minimal infective dose" (m.i.d.). In the second group, the virus suspension of unknown titre is inoculated into a fowl, together with a standard virus suspension of known titre. The titres are compared either by the relative sizes of the tumours ultimately produced or by the time elapsing before detectable tumours arise.

This second group of methods, although useful, no doubt, in certain types of work, was avoided by us. There was no *a priori* reason for assuming that a

constant standard could be obtained or that the subsequent comparison would be unaffected by such factors as the season of the year or the nature of the medium in which the virus was suspended. The first group (" limiting dilution " methods) had, also, the advantage of giving some degree of absolute measure corresponding to the actual number of tumour-producing particles.

Two injection routines have been favoured by previous workers ; either the intradermal or the intramuscular. The former allows several different dilutions to be tested in the same bird, but accurate localization of the injection is rendered difficult by the paper-thin structure of the avian skin. Moreover, the frequent occurrence of resistant fowls necessitates replication of the tests. Intramuscular injections involve a more readily standardized reaction site, but 6-week-old birds frequently show a variable and unpredictable resistance to the virus.

Such resistant birds may not react to 1000 times the virus dose which will produce tumours in susceptible birds, and if the end-point (m.i.d.) is dependent, as may be shown, upon a Poisson distribution of virus particles, then a large number of 6-week-old fowls would be required for an accurate assay.

It is inconvenient to use large numbers of birds of this age for a single assay and for such reasons the use of embryos or of day-old chicks, which may readily be obtained in large numbers, was investigated. Embryos were found to be less convenient than day-old chicks, since a tumour is only produced in the embryo, when (and where) a deliberate injury is inflicted at the time of inoculation (i.e. pock-count methods cannot be used). Moreover, the separate injection of each embryo is a laborious task.

The day-old chick is known to show less virus resistance than older birds (Carr, 1943). Such chicks may be used in large numbers, but, since metastasis to the site of injury produced by a non-infective inoculation may occur, only one test injection in each chick is possible (Carr, 1943).

Preliminary tests on serial decimal dilutions in groups of 50 chicks were encouraging. False negatives in birds receiving high concentrations of virus did not occur, indicating that the chicks were not manifesting resistance to the virus. The nature of the " tailing-off " of activity in the high dilutions indicated that an accurate estimate of the titre could be obtained. Table IV gives examples of assays from a few of the early tests.

TABLE IV.

Experiment number.	Dilution. 1×10^{-3}.	1×10^{-4}.	1×10^{-5}.	1×10^{-6}.	1×10^{-7}.	1×10^{-8}.
1	—	3/3	4/4	4/5	0/5	0/5
2	—	4/4	5/5	6/6	1/7	0/7
3	—	—	6/6	2/6	1/5	0/6
4	8/8	3/7	1/7	0/7	0/7	0/7
5	—	—	6/6	6/6	1/7	0/7

In the dilution columns of these Tables (IV, V and VI) the numerator of the fraction shown refers to the number of tumours and the denominator to the number of inoculations. All titres are referred back to the wet weight of tumour tissue used initially. Thus 10^{0} is a concentration of virus product in suspension

such that 1 ml. ≡ 1 g. tumour (1 ml. g. equivalent), 10^{-5} is thus equivalent to 10^5 infective doses per g. of original tumour.

In practice it was found convenient to inject an inoculum of 0·1 or 0·2 ml. into a leg muscle of these chicks (which were aged 2 to 7 days). The tumours begin to be palpable after about 14 days, and most have appeared at 21 days. The survivors are killed and examined at 28 days. Sudden deaths in the young birds as a result of the "haemorrhagic disease" described by Milford and Duran-Reynals (1943) did not occur when the inoculum was given into the leg muscle.

Irregular results were obtained in the assay when the virus suspension was obviously aggregated (Table V). This virus suspension settled out even under gravity.

TABLE V.

Experiment number.	Dilution. 2×10^{-5}.	2×10^{-6}.	2×10^{-7}.	2×10^{-8}.
6	3/4	5/5	0/2	2/3

It is further obvious that, where the titre of a virus preparation is known approximately (as it now is for the majority of our frozen-dried preparations), the serial dilution may be gauged more closely and the bulk of the 50 chicks used for the important limiting dilutions. The methods for this require the normal statistical treatment and tables for arranging such experiments are given by Fisher and Yates (1947).

Comparison of assay methods in 6-week-old birds and in young chicks.

Several comparisons were made of the titre of preparations given by assay in 6-week-old birds and in young chicks. In each test the young chicks were clearly more sensitive to the virus.

The results are shown in Table VI.

TABLE VI.

Experiment number.	Age of bird.	Factor.	Dilution. 10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	10^{-5}.	10^{-6}.	10^{-7}.	10^{-8}.
7	6 weeks	0·5×	—	4/4	4/4	1/4	0/4	0/4	0/4	—
	Chicks	0·2×	—	—	—	6/7	0/6	0/6	0/6	—
8	6 weeks	0·5×	4/4	1/4	0/4	—	—	—	—	—
	Chicks	0·2×	—	—	2/5	0/7	0/7	0/7	—	—
9	6 weeks	0·5×	2/3	2/2	2/3	2/2	2/3	2/3	0/2	0/2
	Chicks	0·1×	—	—	8/8	8/8	5/8	1/8	1/8	0/8
10	6 weeks	0·5×	4/4	4/4	4/4	4/4	4/4	0/4	0/4	0/4
	Chicks	0·1×	—	5/5	2/2	5/5	4/6	1/6	0/5	—
11	6 weeks	0·5×	4/4	3/4	0/4	0/4	0/4	0/4	—	—
	Chicks	0·2×	—	4/4	6/6	0/6	1/7	0/7	0/7	—

SUMMARY.

A number of methods for the preparation of "concentrates" of Rous agent have been investigated. Direct deposition of the virus from hyaluronidase-treated tumour extracts followed by treatment of the deposit with crystalline

trypsin (which removes more than 60 per cent of the non-virus proteins) gives a product containing only 2 per cent of the nitrogen of the clarified tumour extracts, but possessing almost all the virus activity. The minimal infective dose of such comparatively crude preparations is of the order of 1×10^{-8} mg. N and these virus concentrates may readily be stored by freeze-drying. A biological assay method using very young chicks has been developed. These chicks are found to be more sensitive to the virus than older birds and are obviously more convenient to handle and maintain.

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REFERENCES.

Amies, C. R.—(1937) *J. Path. Bact.*, **44**, 141.
Idem and Carr, J. G.—(1939) *Ibid.*, **49**, 497.
Brunfield, H. P., Stulberg, C. S., and Halvorson, H. O.—(1948) *Proc. Soc. exper. Biol. N.Y.*, **68**, 410.
Bryan, W. R., Riley, V. T., Deihl, D. G., and Voorhees, V.—(1947) *J. nat. Cancer Inst.*, **7**, 447.
Carr, J. G.—(1943) *Brit. J. exp. Path.*, **24**, 127.
Idem and Harris, R. J. C.—(1951) *Brit. J. Cancer*, **5**, 95.
Claude, A.—(1935*a*) *J. exp. Med.*, **61**, 27.—(1935*b*) *Ibid.*, **61**, 41.—(1937) *Ibid.*, **66**, 59.—(1938) *Science*, **87**, 467.—(1939) *Ibid.*, **90**, 213.—(1940) *Ibid.*, **91**, 77.
Idem and Rothen, A.—(1940) *J. exp. Med.*, **71**, 619.
Cox, H. R., van der Scheer, J., Aiston, S., and Bohnel, E.—(1947) *J. Immunol.*, **56**, 149.
Dmochowski, L.—(1948*a*) *J. nat. Cancer Inst.*, **9**, 57.—(1948*b*) *Ibid.*, **9**, 69.
Fischer, R. G.—(1949) *Proc. Soc. exper. Biol., N.Y.*, **72**, 323.
Fisher, R. A., and Yates, F.—(1947) 'Statistical Tables for Biological, Agricultural and Medical Research.' 3rd Edn. London (Oliver & Boyd).
Ledingham, T. C. G., and Gye, W. E.—(1935) *Lancet*, i, 376.
McIlvaine, T. C.—(1921) *J. Biol. Chem.*, **49**, 183.
McIntosh, J.—(1935) *J. Path. Bact.*, **41**, 215.
Milford, J. J., and Duran-Reynals, F.—(1943) *Cancer Res.*, **3**, 578.
Moyer, A. W., Sharpless, G. R., Davies, M. C., Winfield, K., and Cox, H. R.—(1950) *Science*, **112**, 459.
Pirie, A.—(1933) *Biochem. J.*, **27**, 1894.
Pollard, A.—(1938) *Brit. J. exp. Path.*, **19**, 124—(1939) *Ibid*, **20**, 429.
Riley, V. T.—(1948) *Science*, **107**, 573.
Shemin, D., and Jobling, J. W.—(1940) *J. exp. Med.*, **72**, 697.
Idem and Sproul, E. E.—(1942) *Cancer Res.*, **2**, 514.
Stanley, W. M.—(1945) *J. exp. Med.*, **81**, 193.
Stern, K. G., and Duran-Reynals, F.—(1939) *Science*, **89**, 609.
Sturm, E., and Duran-Reynals, F.—(1932) *J. exp. Med.*, **56**, 711.
Wagner, J. C., Golub, O. J., and Andrew, V. W.—(1948) *Proc. Soc. exp. Biol., N.Y.*, **69**, 202.

PRESERVATION OF THE AGENT OF THE ROUS SARCOMA NO. 1 BY FREEZE-DRYING.

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IT has long been known that the Rous sarcoma may be transmitted by dried tumour tissue and it has been concluded, therefore, that the tumour agent can survive under such conditions (Rous and Murphy, 1914; Rous, 1911; and Hoffstadt and Tripi, 1946). Knox (1939) was able to dry Berkefeld filtrates of both Rous sarcoma and Fujinami myxosarcoma, but only with some attendant loss of activity. The filtrates were frozen in a thin shell on the inner surface of ampoules by immersing these in an acetone-solid carbon dioxide bath. The ampoules were then connected to a manifold and the ice sublimed *in vacuo*. Knox's (1939) apparatus and conditions were later used by Dmochowski (1948) for the freeze-drying of Rous virus suspensions produced by fractional centrifugation and other methods. The products, reconstituted, after 14 days, to the original volume with water, were invariably inactive. Dmochowski (1948) claimed that similar virus suspensions treated with a drop of fowl or rabbit serum suffered no loss of activity under the same conditions, but that addition of salts, such as NaCl, KCl or $CaCl_2$, had no such preservative action. No data were presented relevant to the question either of the concentration of agent in the suspension dried or of the degree of exactitude of the assay procedure used. It was further claimed that addition of 1:10,000 HCN before drying protected the virus, in that 60 to 80 per cent of the initial activity could be recovered. It is difficult to accept the hypothesis that the virus particles may be damaged mechanically by being tightly packed together during drying or that the loss of activity may be explained by rapid oxidation processes during drying, since drying from the frozen state takes place *in vacuo* and at very low temperatures. The possibility that the freezing of the virus suspension was the inactivating stage does not appear to have been investigated.

The preservation of other animal viruses by freeze-drying has presented essentially similar problems. In general, suspensions of virus-infected tissues or body-fluids are readily preserved (Scherp, Flosdorf and Shaw, 1938; Sawyer, Lloyd and Kitchen, 1929; Shope, 1931; Johnson and Goodpasture, 1934; and Kitchen, 1934). However, dilute purified suspensions of such viruses, as well as solutions of biologically-active proteins, are unstable and are rapidly inactivated under conditions in which the total protein content of the suspension or solution is low (Adams, 1948; Bauer and Pickels, 1940). The Rous agent behaves in exactly the same way and, moreover, the rate of inactivation is proproportional to the temperature; the higher the temperature, the more rapid

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the rate of inactivation (Claude, 1937*a*, 1937*b*; Claude and Rothen, 1940). Adams (1948) has shown that for the *E. coli* phage series T_1–T_7 this inactivation is a surface phenomenon. A dilute suspension of T_7 phage (10^4 per ml.) in buffered saline at pH 6·5 was totally inactivated at 26° C. by gentle shaking for 35 minutes. If the shaking was carried out in the absence of gas space, albeit with glass beads in the tube, inactivation was negligible. Adams (1948) found that as little as 0·01 γ per ml. of gelatin had a protective effect and 1 γ per ml. gave complete protection for T_5 phage for 14 minutes. The duration of the protective effect was found to be a function of the gelatin concentration, since the gelatin itself is "denatured." Protection could also be obtained with gum arabic or with serum albumin although 100-fold and 10-fold the gelatin concentration were required.

This work may well provide the theory behind the observation that viruses are more stable when diluted in serum or broth than when the dilutions are made with salt or water. Equally one might expect that small viruses would be inactivated more rapidly than larger, and that the rate of inactivation would be proportional to temperature, since the forces responsible for bringing the particles to an interface will be thermal forces.

Such conditions as these have frequently been realized empirically. Rivers and Ward (1935) sought to add a material to vaccinia virus suspension which would (*a*) act as a protective agent, (*b*) add bulk to the final product and (*c*) go back into solution with ease, carrying the virus with it. Egg albumin fulfilled these conditions, but was antigenic and 2·5 per cent gum acacia was substituted with considerable success.

Behrens and Ferguson (1936) made similar observations on the same virus. They studied, as protective agents, peptone (0·1 per cent and 1·0 per cent), glycerol (25 per cent and 50 per cent), inactivated rabbit serum (10 per cent), gelatin (0·1 per cent), isotonic glucose and 1:1000 and 1:2000 neutralized cysteine hydrochloride. Of these, 1 per cent peptone and 0·1 per cent gelatin gave maximum preservation.

Schade (1945) found that phage suspensions containing initially 1×10^9 active particles per ml. and frozen-dried without protection were reduced after reconstitution to 1×10^3 particles per ml. Addition of Difco meat extract before drying resulted in a final count of 1×10^5 per ml., whereas extracts of fresh brain, kidney or pancreas afforded complete protection.

The problem was that of preserving the Rous agent in *bulk* in a form suitable for chemical and biochemical investigation and with virtually unreduced biological activity.

The protective agent was sought with optimum properties: (*a*) be readily separable from the virus, (*b*) provide a reticulum at the drying stage, such that drying takes place rapidly, (*c*) prevent virus aggregation, (*d*) have antioxidant properties.

MATERIALS AND METHODS.

The results of these investigations, which extended over a long period, are summarized in the following tables. The methods by which the initial virus concentrates were produced were common throughout and, to simplify the tables, the following abbreviations will be used to describe the purification of the virus prior to freeze-drying and after the subsequent rehydration.

(1) *Preparative methods.*

The initial virus concentrates were prepared throughout by macerating (Waring blendor) fresh or frozen-and-thawed tumour tissue in 10 volumes of 0·005 M phosphate buffer, pH 7·2, containing rat testis extract and 1:10,000 HCN. This suspension was clarified on the Sharples centrifuge and the virus deposited from the supernatant on to a cellophane bowl lining sheet by deposition at high speed (Carr and Harris, 1951). This virus-containing deposit was then resuspended in a volume of medium (concentration medium) equal v/w to the original weight of tumour tissue.

(2) *Concentration media.*

(a) *Buffer-trypsin*, B-T.—McIlvaine's (1921) phosphate-citric acid buffer (pH 7·5 to 8·0) prepared from 0·2 M $Na_2HP_1O_4$ and 0·1 M citric acid and diluted 1:19 with water (buffer) and a few mg. per cent of crystalline trypsin added.

(b) *Lemco-trypsin*, L-T.—Lemco broth (Baird and Tatlock, Ltd.) to which a few mg. per cent of crystalline trypsin was added.

(c) *Lemco : Dextrose*, L-D.—1:1 v/v Lemco broth : 5 per cent dextrose.

(d) *Buffered Lemco : Dextrose*, B.L:D.—As (c), adjusted to pH 7·5 with 0·2 M sodium phosphate.

(e) *Buffered Lemco : trypsin*, B.L-T.—1:1 v/v Lemco broth : B-T.

(f) *Buffered Lemco : Dextrose-trypsin*, B.L:D-T.—As (d), to which a few mg. per cent crystalline trypsin was added.

(3) *Concentration methods prior to freeze-drying.*

Method I.—Deposition of virus from suspension in the "concentration medium" on Serval Angle Centrifuge SS1 at 0° to 5° C., 11,000 r.p.m. (= 14,500 g.) for 55 minutes.

Method II.—Clarification of the suspension in the "concentration medium" under the same conditions as Method I, but at 5000 r.p.m. (= 3000 g.) for 20 minutes, followed by deposition of virus from the supernatant (Method I).

(4) *Freeze-drying medium.*

Virus pellets from Methods I or II were resuspended in a volume of medium equal to the original weight of tumour. Aliquots of 10 ml. were frozen-dried in 25 ml. McCartney bottles.

(5) *Freeze-drying.*

A laboratory-scale freeze-drier (Edwards' Centrifugal, Type 3) was used throughout the work and the conditions under which the freeze-drying was carried out were as uniform as possible. The McCartney bottles were accommodated in drilled compartments in the centrifuge head of the drying chamber of the machine. Each bottle was inclined towards the axis of rotation, so that when the head was spinning the contents of each bottle formed a wedge.

The virus suspensions were snap-frozen by evaporative cooling, and the purpose for spinning the bottle was 2-fold. (*a*) Frothing was prevented and (*b*) the wedge of frozen material presented a greatly increased surface area with a consequent increase in the rate of drying. The water vapour sublimed from the ice was collected on coils refrigerated to a temperature of — 40° to — 50° C.

The drying chamber was maintained at room temperature at which the final dry product was held. The rate of drying was high throughout the drying cycle and no evidence was found to suggest that, as the layer of dried virus material increased in thickness, the rate of evaporation of vapour fell off and the underlying frozen mass melted (Bauer and Pickels, 1940).

The drying cycle occupied 40 to 44 hours. The bottles, which were either uncapped, or lightly capped with two or three layers of sterile surgical gauze during the drying, were then screw-capped and sealed with tape and stored at — 2° C. to — 4° C.

(6) *Resuspension of frozen-dried material.*

A volume of medium one to two times the volume of water removed was added to the frozen-dried mass and resuspension completed by recapping the bottle and shaking gently. The media used, and here abbreviated, included :

(a) *Buffered-saline*, B-S.—Ten per cent saline adjusted to pH 7·2 with 0·2 M sodium phosphate.

(b) *Buffer, pH* 5·0.—McIlvaine's phosphate-citric acid, diluted 1:19 with water.

(c) *Water-trypsin*, W-T.—Solution containing a few mg. per cent crystalline trypsin and adjusted to pH 7·5.

(d) *Glycerol-phosphate*, G-Ph.—1:2 v/v 50 per cent glycerol ; 0·005 M phosphate buffer at pH 7·2.

(7) *Resuspension methods.*

The rehydrated material was rarely assayed without a further purification procedure.

A : (i) Deposition of the virus in the high-speed angle head of the International Refrigerated Centriguge, PRI, running at 0° C., 15,000 r.p.m. (= 17,000 g.) for 57 minutes. The virus pellet was then redispersed in an equal volume of cooled Lemco broth by gentle agitation with a pipette, followed by—

(ii) Clarification (to remove aggregated material) in the same centrifuge. 5000 r.p.m. (= 2000 g.) for 18 minutes. The supernatant was then assayed.

B : As A, but cooled Tyrode's solution used for resuspension of the pellets.

C : Step Aii only. Supernatant then assayed.

D : As A, but Ai preceded by an additional clarification step Aii—thus Aii, Ai, Aii.

E : As D, but omitting the final clarification, thus, Aii, Ai.

F : As D, but 10 per cent saline used instead of Lemco broth.

G : As D, but substituting L-T.

H : As D, but substituting B-S.

I : E repeated once with intermediate resuspension in 0·005 M phosphate buffer at pH 7·2.

Assay procedure.

The final suspension from procedures A to I was serially diluted in Lemco broth, such that 10° corresponded to the virus equivalent of 1 g. of original tumour tissue in 1 ml. broth. Virus was assayed by the procedure described in a previous paper (Carr and Harris, 1951). The figures given in Table I relate to the maximum dilution at which tumours were found to be present

macroscopically at autopsy after an interval of not more than 35 days. Where figures are given in a column—Assay (fresh)—these refer to an assay of the virus immediately prior to freeze-drying and using the *same* suspension. The column, Freeze-drying Interval, gives the number of days which elapsed between the beginning of the freeze-drying cycle and the date of assay of the rehydrated virus.

RESULTS.

Freeze-drying in water (Table I).

The Sharples deposits, obtained from a suspension containing 1:10,000 HCN, were resuspended in various media and concentrated. The final virus-containing pellets were resuspended in water and the suspension immediately frozen-dried. Assay (fresh) figures were not invariably obtained but, in general, and applicable to the whole of the experiments presented here, these figures, where determined, varied between 10^{-6} and 10^{-8}, with occasional "lapses" to 10^{-5}, i.e. 1 ml. g. equivalent of fresh virus suspension contained between 10^{6} and 10^{8} minimal infective doses of virus.

TABLE I.—*Water.*

Date.	Concentration medium (2).	Concentration method (3).	Interval after freeze-drying (days).	Resuspension medium (6).	Resuspension method (7).	Assay (frozen-dried).
9.ii.49	Water	None	2	B-T	E (twice)	10^{-3}
15.ii.49	„	„	3	„	E („)	10^{-3}
22.ii.49	„	„	2	„	E („)	10^{-5}
5.iv.49	„	„	2	„	E	$<10^{-5}$
5.iv.49	„	„	2	0·1 satd. Na_2CO_3 and trypsin	E	Nil
4.i.49	B-T	I	2	Water	None	10^{-2}
1.ii.49	„	I	6	„	„	10^{-2}
5.vii.49	„	II	2	10% saline	E	10^{-2}
18.viii.49	„	II (twice)	8	B-S	E	10^{-1}
22.iii.49	W-T	None	7	Lemco	None	10^{-7}
5.iv.49	Water	„	2	„	E	10^{-5}
29.iii.49	B-T	I	2	„	E	10^{-5}

Table I demonstrates that although the virus was still recoverable after freeze-drying in water, the titre, with notable exceptions, was much reduced. Suspensions which had received little purification prior to freeze-drying (where Column 3 reads "None") showed better survival rates than those which had been treated with trypsin and fractionally centrifuged before freeze-drying (4.i.49, 1.ii.49, 5.vii.49, 18.viii.49). It appeared advantageous, therefore, to carry out the bulk of the purification procedures after rehydration. This may be an example therefore, of protection by added protein. The striking results are those of 22.iii.49, 5.iv.49 and 29.iii.49, where it seems that Lemco broth can reactivate (disaggregate ?) inactive virus. A similar result appears from Table VII if the result of 29.iii.49 is compared with that of 5.vii.49, and from Table VIII, 15.iii.49 being compared with 29.iii.49; although activation was *not* found in the experiments with egg albumin or egg white.

Freeze-drying in salt solutions (Table II).

The Sharples deposits were resuspended throughout in 0·005 M phosphate buffer, pH 7·2, containing a few mg. per cent of crystalline trypsin (except

12.vii.49, where trypsin was not added). The suspensions were not further fractionated in 4 cases, but were fractionated in 6, and the virus-containing pellets resuspended in various media and frozen-dried.

TABLE II.—*Salt Solutions.*

Date.	Concentration method (3).	Freeze-drying medium (4).	Interval after freeze-drying (days).	Resuspension medium (6).	Resuspension method (7).	Assay (fresh).	Assay (frozen-dried).
15.iii.49	None	B-T	2	Water	E (twice)	10^{-7}	10^{-1}
11.iv.49	„	„	2	„	None	—	Nil
20.iv.49	„	„	8	„	„	10^{-5}	10^{-1}
12.vii.49	„	Buffer	2	„	C	—	10^{-2}
29.iii.49	II	„	2	Lemco	E	—	$<<10^{-5}$
19.vii.49	II	„	9	B-S	E	10^{-8}	10^{-1}
19.vii.49	II	„	9	G-Ph	E	10^{-8}	10^{-3}
19.vii.49	II	„	15	7% saline	E	10^{-8}	10^{-3}
26.vii.49	II	„	2	B-S	E	10^{-8}	10^{-1}
26.vii.49	II	„	2	G-Ph	E	10^{-8}	10^{-3}

Table II shows that sodium phosphate concentrations as low as 0·005 M have an inactivating effect upon the virus. Recoveries were very small in every case and Lemco had no " reactivating " action under these conditions.

Freeze-drying in Lemco : dextrose. (Table III).

The Sharples deposits were resuspended in various media, and in most cases fractionated centrifugally. The virus pellets were resuspended in 1:1 Lemco broth : 5 per cent dextrose (except on 1.xi.49 and 15.xi.49, when the mixture was adjusted to pH 7·5 with 0·2 M sodium phosphate) and frozen dried.

TABLE III.—*Lemco : Dextrose.*

Date.	Concentration medium (2).	Concentration method (3).	Interval after freeze-drying (days).	Resuspension medium (6).	Resuspension method (7).	Assay (frozen-dried).
13.x.49	B.L:D-T	II	15	Water	A*	10^{-4}
19.x.49	„	II	9	„	A*	10^{-5}
1.xi.49	„	II	7	„	A*	10^{-1}
15.xi.49	B-T	II	9	„	A	10^{-2}
27.x.49	L:D	None	2	W-T	A	10^{-5}
27.x.49	B.L:D	„	2	„	A	10^{-4}
9.viii.49	B-T	I	3	B-S	C	10^{-5}
9.viii.49	„	I	5	„	C	10^{-6}
29.ix.49	„	II	7	„	A	10^{-3}

* Deposited virus pellets resuspended in 1:1 Lemco : 5 per cent dextrose instead of Lemco alone.

These results are variable ; 4 of 9 experiments showed a final titre of 10^{-5} or more. There appeared to be no advantage in using a broth-containing medium in the pre-freeze-drying processing and no difference in the result if the trypsin treatment was carried out before or after the desiccation. The use of the broth alone gave much better results and the use of a mixture was therefore discontinued.

Freeze-drying in Lemco-Trypsin (Table IV).

The Sharples deposits were resuspended in Lemco broth, a few mg. per cent crystalline trypsin added and the suspension immediately frozen-dried.

TABLE IV.—*Lemco-Trypsin.*

Date.	Interval after freeze-drying (days).	Resuspension medium (6).	Resuspension method (7).	Assay (frozen-dried).
28.vi.49	21	10% saline	G	10^{-1}
28.vi.49	21	B-S	G	10^{-3}
14.iii.50	3	Water	D	10^{-6}
14.iii.50	3	1:10^4 Lissapol	D	10^{-6}
21.iii.50	2	Water	C	10^{-4}
4.iv.50	2	,,	D	10^{-5}
13.iv.50	15	,,	E	10^{-6}
2.v.50	2	,,	D	10^{-5}
9.v.50	9	,,	E	10^{-5}
22.vi.50	5	,,	D	10^{-3}
29.vi.50	6	,,	D	10^{-5}

These suspensions were " self-protecting " to a large extent because they were unfractionated before freeze-drying. Two points emerge. First, the use of 10 per cent saline for rehydration, either alone or buffered to pH 7·2, was disadvantageous—of the other 9 experiments, 7 gave a titre of 10^{-5} or better. Second, the use of 1:10,000 Lissapol for rehydration had no deleterious effect upon the virus.

Freeze-drying in Lemco (Table V).

The Sharples deposits were resuspended in B-T and " purified " by fractional centrifugation. The virus pellets were then resuspended in Lemco broth and frozen-dried.

TABLE V.—*Lemco.*

Date.	Concentration method (3).	Interval after freeze-drying (days).	Resuspension medium (6).	Resuspension method (7).	Assay (fresh).	Assay (frozen-dried).
29.iii.49	I	3	Water	A	—	10^{-5}
5.vii.49	II	157	,,	B	10^{-6}	10^{-6}
5.vii.49	II	2	10% saline	C	10^{-6}	10^{-7}
9.viii.49	I	36	B-S	C	10^{-6}	10^{-6}
10.xi.49	I	7	Water	D	10^{-5}	10^{-6}
7.ii.50	I	10	,,	A	—	10^{-6}
22.xi.49	II	2	,,	A	10^{-5}	10^{-4}
28.xi.49	I	18	,,	D	—	10^{-4}
6.xii.49	II	16	,,	D	—	10^{-6}
14.xii.49	II	2	,,	D	—	10^{-6}
22.ii.50	II	2	,,	D	—	10^{-5}
28.ii.50	II	2	,,	D	—	10^{-6}
18.iv.50	II	17	,,	D	—	10^{-4}
16.v.50	II	3	,,	D	—	10^{-5}
25.ix.50	II	4	,,	C	—	10^{-4}

With the exception of two differences in the nature of the resuspending medium, the conditions for these 15 experiments are almost identical. In at least 2 cases the titre of the recovered virus was higher than that of the fresh

material. In 11 of the 15 experiments the titres were 10^{-5} or better, and it is also apparent that the titre bears no direct relationship to the interval between freeze-drying and rehydration and assay.

In other experiments (Table VI) the Sharples deposits were resuspended in various media all but one of which contained broth, and, in the majority of experiments tabulated, the suspension was frozen-dried without further "purification."

The variations were produced in the nature of the resuspending media.

TABLE VI.—*Lemco.*

Date.	Concentration medium (3).	Concentration method (4).	Interval after freeze-drying (days).	Resuspension medium (6).	Resuspension method (7).	Assay (frozen-dried).
26.iv.49	Lemco	None	63	10% saline	F	10^{-4}
26.iv.49	„	„	60	„	C	10^{-4}
26.iv.49	„	„	60	0·9% saline	C	10^{-4}
26.iv.49	„	„	60	0·2 M sodium phosphate	C	10^{-4}
26.iv.49	„	„	60	Buffer pH 5·0	C	$<<<10^{-4}$
26.i.50	„	„	5	W-T	C	10^{-5}
7.iii.50	„	„	2	Water	D	10^{-6}
4.iv.50	„	„	79	Buffer pH 9·0	A	10^{-4}
4.iv.50	„	„	79	Buffer pH 9·0 and trypsin	A	10^{-5}
8.vi.50	„	„	5	Water	D	10^{-4}
28.iii.50	L-T	II	21	„	C	10^{-5}
28.iii.50	„	II	21	„	C	10^{-4*}
25.iv.50	Buffer	II	3	„	E	10^{-5}
23.v.50	1:1 Lemco: Buffer-T	II	2	„	D	10^{-3}

* Freeze-dried in 10 per cent Lemco.

The results show that the use of buffer or salt solution for rehydration was not advantageous. In 5 of 14 experiments there was a titre of 10^{-5} or higher, and in 4 of these the deposit had been resuspended in water or water-trypsin. The other had been treated with trypsin at pH 9·0. All except one (23.v.50) of the experiments in which trypsin was used either before or after the desiccation gave good end-products. The effect of trypsin may, indeed, be estimated directly from the comparison experiments of 4.iv.50. It is interesting to see also that on 28.iii.50, where Lemco is compared with 10 per cent Lemco as a medium for freeze-drying, there is a reduction in the titre with the weaker broth.

Freeze-drying in carbohydrate media (Table VII).

The Sharples deposits were resuspended in buffer-trypsin and "purified" in the usual way. The final virus-containing pellets were resuspended in various carbohydrate media and frozen-dried.

With the exception of the experiment (29.iii.49) in which the frozen-dried material was rehydrated with Lemco broth, and of 5.vii.49, where 0·125 per cent dextrose was used, the results of this series are uniformly poor. There is a suggestion that dextrose has an optimum action at a concentration of about 3 per cent, and in this group (18.viii.49) the virus suspensions had been considerably "purified" before freeze-drying.

TABLE VII.—*Carbohydrate Media.*

Date.	Concentration method (3).	Freeze-drying medium (4).	Interval after freeze-drying (days).	Resuspension medium (6).	Resuspension method (7).	Assay (fresh).	Assay (frozen-dried).
29.iii.49	I	Starch (0·1%)	3	Lemco	A	$>10^{-7}$	10^{-5}
5.vii.49	II	„ (0·125%)	2	10% saline	C	10^{-6}	10^{-2}
1.xi.49	II	Cellofas A* (1%)	7	Water	D	10^{-5}	Nil
10.xi.49	II	„ A* (0·3%)	7	„	D	10^{-5}	10^{-1}
5.vii.49	II	Dextrose (0·125%)	2	10% saline	C	10^{-6}	10^{-5}
9.viii.49	I	„ (5%)	3	B-S	C	10^{-6}	10^{-1}
9.viii.49	I	„ „	3	Water	C	10^{-6}	10^{-1}
18.viii.49	II (twice)	„ „	19	B-S	A	—	10^{-1}
18.viii.49	„	„ „	8	„	E	—	10^{-1}
18.viii.49	„	„ (3%)	8	„	E	—	10^{-3}
18.viii.49	„	„ „	19	„	A	—	10^{-4}
18.viii.49	„	„ (1%)	8	„	E	—	10^{-2}

* By courtesy of Imperial Chemical Industries, Ltd.

Freeze-drying in protein or protein hydrolysate media (Table VIII).

The Sharples deposits were treated in a manner identical with those of the last (carbohydrate) series.

TABLE VIII.—*Protein or Protein Hydrolysate Media.*

Date.	Concentration method (3).	Freeze-drying medium (4).	Interval after freeze-drying (days).	Resuspension medium (6).	Resuspension method (7).	Assay (fresh).	Assay (frozen-dried).
10.xi.49	II	1:2 guinea-pig serum: 0·9% saline	40	Water	D	10^{-5}	10^{-4}
29.iii.49	I	Egg albumin (0·1%)	3	Lemco	A	$>10^{-7}$	$<10^{-5}$
29.iii.49	I	Egg white, 1% in 0·9% saline	3	„	A	$>10^{-7}$	$<10^{-5}$
15.iii.49	I	Gelatine (0·1%)	2	Buffer	I	10^{-7}	10^{-4}
29.iii.49	I	„	3	Lemco	A	$>10^{-7}$	10^{-5}
26.i.50	None*	6% gelatine hydrolysate† in 0·9% saline at pH 7·2	5	Water	H	—	10^{-2}
7.ii.50	I	0·2% gelatine hydrolysate as above	10	„	A	—	10^{-2}
10.xi.49	II	1:1 protein hydrolysate (W):5% dextrose	45	„	D	10^{-5}	10^{-4}
10.xi.49	II	Protein hydrolysate‡ (Wellcome)	7	„	D	10^{-5}	10^{-6}
10.xi.49	II	Peptone (B.D.H.) (1%)	40	„	D	10^{-5}	10^{-4}
29.ix.49	I	Peptone (Chapoteaut)‡ (1%)	7	B-S	A	10^{-6}	Nil
5.vii.49	II	Neutralized cysteine HCl (0·125%)	2	10% saline	C	10^{-6}	10^{-2}
10.xi.49	II	B.A.L. (0·1%)	45	Water	D	10^{-5}	10^{-2}
10.xi.49	II	B.A.L. (0·05% in 2·5% dextrose)	7	„	D	10^{-5}	10^{-5}

* Sharples deposit resuspended directly in freeze-drying medium.
† Knox Gelatin Protein Product Inc.
‡ By courtesy of Dr. Linggood.

The results show that intact protein media possessed no advantage over Lemco broth. Egg albumin, egg white and gelatin gave poor recoveries and the products were difficult to reconstitute. A minimal preservation action towards the Rous virus was obtained from 0·2 per cent gelatin hydrolysate, which had been used successfully by McCullagh, Cassidy, Valentine and Tolksdorf (1949) for the preservation of purified testicular hyaluronidase. Peptone (B.D.H., 1 per cent) and a protein hydrolysate from Burroughs Wellcome & Co., Ltd., had good properties, but another peptone (Chapoteaut) caused complete inactivation. Neither 0·1 per cent B.A.L. or 0·125 per cent neutralized cysteine hydrochloride gave protection, and the protection afforded by 2·5 per cent dextrose containing 0·05 per cent B.A.L. was almost certainly the effect of the sugar.

DISCUSSION.

The object of this work was to find suitable conditions for the preservation of fully active Rous virus on a large scale, the requirements being that the resuspended product should be non-aggregated and capable of being further purified by fractional centrifugation. The method now adopted, that of freeze-drying virus concentrates in Lemco broth, fulfils the condition, but little light has been thrown on the mode of action of the stabilizing material. Virus concentrates dried in this way have retained their full activity for minimum periods of 12 months.

The experiments with water alone showed that Dmochowski's (1948) results are confirmed in so far as virus may be recovered after drying—and our preparations had all been treated with 1:10,000 HCN during the isolation procedure—but it was not possible under these conditions to recover as much as 60 to 80 per cent of the initial virus activity as he claimed.

Salt solutions had no preservative action, and broth-containing media showed immediate advantages, even where the suspensions had been " purified " by centrifugal fractionation before desiccation. The action of the broth appears to be confined to the freezing-and-drying cycle or subsequently, since nothing was gained by carrying out the concentration stages in broth or broth-containing media, for which buffer-trypsin is quite satisfactory. It is unlikely, therefore, that the effect is solely an example of the phenomenon described by Adams (1948). The conditions of freeze-drying, moreover, do not involve vigorous shaking of the material but, equally, there is as yet no knowledge of the surface conditions which exist at an ice surface which is gradually receding through a drying mass. The observation that weaker (10 per cent) Lemco broth is a less effective protective agent is of interest in this respect.

It is possible to suppose that the action of broth and similar material is complex, that one or a combination of such factors as the following are important: (*a*) The broth provides optimum conditions during freezing—such as an optimum eutectic point; (*b*) that protection is conferred against the deleterious effect of heavy metals or of oxidation systems; (*c*) that, in some way, the tendency of the virus to aggregate is reduced; or (*d*) that an optimum amount of water only is lost. The interesting suggestion has been made that the preservative action of glycerol and sugar solutions in general is a result of water retention in or around the virus or bacterium (Kaiser, 1942). There appear to be no data available for the degree of dryness which has been achieved with other dried viruses. It may

well be that it is possible to remove *irreversibly* water which is part of the internal structure of the body, with resultant inactivation.

Three, (*a*), (*b*) and (*d*), of the above four suggestions are rendered less probable by the fact that it appears to be possible to "reactivate" virus dried under unfavourable conditions by resuspending the dried material in Lemco broth.

The mode of action of broth and similar media is being further investigated.

SUMMARY.

The preservation of Rous virus activity has been investigated after freezing-and-drying virus concentrates in a variety of media. In general, less highly purified suspensions are more readily preserved, and the tumour protein impurities apparently have a protective action. Broth-containing media have given optimum results, and full activity of the dried virus may be recovered after storage for periods of a year or more.

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REFERENCES.

Adams, M. H.—(1948) *J. gen. Physiol.*, **31**, 417.
Bauer, J. H., and Pickels, E. G.—(1940) *J. exp. Med.*, **71**, 83.
Behrens, C. A., and Ferguson, W. W.—(1935) *J. infect. Dis.*, **56**, 84.
Carr, J. G., and Harris, R. J. C.—(1951) *Brit. J. Cancer*, **5**, 83.
Claude, A.—(1937*a*) *J. exp. Med.*, **66**, 59.—(1937*b*) *Amer. J. Cancer*, **30**, 742.
Idem and Rothen, A.—(1940) *J. exp. Med.*, **71**, 619.
Dmochowski, L.—(1948) *J. nat. Cancer Inst.*, **9**, 73.
Hoffstadt, R. E., and Tripi, H. B.—(1946) *J. infect. Dis.*, **78**, 183.
Johnson, C. D., and Goodpasture, E. W.—(1934) *J. exp. Med.*, **59**, 1.
Kaiser, M.—(1942) *Biol. Gen. (Vienna)*, **16**, 513.
Kitchen, S. F.—(1934) *Amer. J. trop. Med.*, **14**, 457.
Knox, R.—(1939) *J. Path. Bact.*, **49**, 467.
McCullagh, D. R., Cassidy, J. W., Valentine, F., and Tolksdorf, S.—(1949) *Proc. Soc. exp. Biol., N.Y.*, **71**, 295.
McIlvaine, T. C.—(1921) *J. biol. Chem.*, **49**, 183.
Rivers, T. M., and Ward, S. M.—(1935) *J. exp. Med.*, **62**, 549.
Rous, P.—(1911) *Ibid.*, **13**, 397.
Idem and Murphy, J. B.—(1914) *Ibid.*, **19**, 52.
Sawyer, W. A., Lloyd, W. D. M., and Kitchen, S. F.—(1929) *Ibid.*, **50**, 1.
Schade, A. L.—(1945) 'Amer. Ass. Adv. Sci. Res. Conference on Cancer,' p. 58.
Scherp, H. W., Flosdorf, E. W., and Shaw, D. R.—(1933), *J. Immunol.*, **34**, 447.
Shope, R. E.—(1931), *J. exp. Med.*, **54**, 373.

CARCINOGENESIS IN PARABIOTIC RATS.

Tumours of Liver and Seminal Vesicle Induced by Acetylaminofluorene in Normal Males Joined to Castrated Males or Females.

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Several methods are available for studying the effect of prolonged stimulation of the sex organs by gonadotropic hormones: (1) repeated implantation of pituitaries obtained from castrates, (2) implantation of testis or ovary into the spleen of a castrated animal (Biskind and Biskind, 1944, 1945) and (3) parabiotic junction of a normal animal to a gonadectomized litter-mate.

The first method is not very effective owing to the generally short survival of the grafted pituitaries. The second method creates an endocrine imbalance through the rapid inactivation of the sex hormones by the liver. The pituitaries of such animals react to the lack of sex hormones in the general circulation with increased output of gonadotropins like the pituitary of a castrate. The ingenious method of the Biskinds (1944, 1945), however, does not allow one to study how the increased hormonal output of the stimulated gonads affects the accessory sex organs, whereas in parabiotic animals this effect can be observed. When a castrate is joined in parabiosis to a normal partner the gonadotropic hormones of the castrate's pituitary pass over into the twin partner and stimulate its gonads, which not only enlarge but also secrete excessive amounts of sex hormones (Martins, 1929). In males these steroid hormones are so quickly metabolized that only the intact partner is exposed to their actions, whereas in females exceptions to this rule can occur (Zeckwer, 1946). Therefore in such experiments the role of the castrate is limited to that of a donor of gonadotropic hormones. This paper describes the results obtained in parabiotic rats, the normal male partner of which received 2-acetylaminofluorene (A.A.F.). We have found in the literature only one reference to an investigation in which a similar experimental procedure has been used. Strömbeck and Ekman (1949) fed a diet containing A.A.F. to parabiotic rats, one of which had been nephrectomized, and obtained in the liver neoplastic lesions which were more pronounced in the partner with intact kidneys.

METHODS.

The rats used belonged to a strain of Albino rats originally derived from the Wistar stock. At the age of 4 to 6 weeks litter-mates of approximately equal size were joined together, using the technique described by Jacobsohn (1948). At the same time one of the partners was castrated. Where an intact male and a spayed

female were joined ovariectomy was performed a week previously. The result of the operation is a pair of rats with a peritoneal cavity common to both. A breakdown of the junction is a rare event.

Operative mortality was practically *nil*, but in the second or third week after the operation a great number of pairs died due to incompatibility of the partners. No pair showing signs of " parabiotic intoxication " survived longer than 5 weeks. The use of more closely inbred rats (three generations of brother-sister mating) did not lower the rate of mortality at this critical period.

In most experiments acetylaminofluorene was given by stomach tube to the normal partner, who received the carcinogen in doses of 4 mg. dissolved in 1 c.c. of peanut oil. The treatment with the carcinogen was started 3 to 4 weeks after parabiosis had been established, when consistent gain in weight and the good condition of the animals showed that the operation had been successful. The A.A.F. was given 3 times weekly and 17 to 38 doses were administered. In one experiment 6 doses of 2 mg. of A.A.F. were given on 6 consecutive days by stomach tube and in another the carcinogen was given to the twins with their diet (4 mg. daily to each rat for a period of 4 weeks). All the rats which received A.A.F. by stomach tube were fed the ordinary stock diet, consisting of meat meal, pollard, bran, maize meal, bone flour and whole wheat supplemented by cabbage. The pair to which the A.A.F. was administered with their food were kept for 4 weeks on a diet consisting of skimmed milk and whole meal flour supplemented by cod-liver oil and cabbage. Subsequently they received the same food as the other pairs.

The twins were killed when a tumour was suspected or when loss of weight indicated that they were in a precarious state of health. Pituitary, testis, accessory sex organs, breast, suprarenal and liver of most pairs were studied histologically. The pituitaries were fixed in mercury-saline (6 per cent), the other organs in Zenker or neutralized formol-saline. For staining of the pituitaries a modified Papanicolau technique was used, the other organs were stained with hæmatoxylin-eosin or according to Weigert-Van Gieson.

The material presented consists of 20 pairs of parabiotic rats treated with A.A.F. and of 6 pairs which did not receive the carcinogen. In 13 pairs a normal male was joined to a castrate and in 5 pairs to a spayed female. The remaining 2 pairs consisted of litter-mates, none of which had been castrated. Of the 6 pairs not treated with A.A.F., 4 were combinations of a normal male joined to a castrated brother, while in the other 2 the male was joined to a spayed female.

In addition, " single " rats were treated with A.A.F. in a similar manner as the normal partners of parabiotic pairs. Five young males received by stomach tube 15 doses of the carcinogen and to a group of 10, 24 doses of A.A.F. were given. These rats were killed when a tumour appeared to be present, or during the 52nd week of the experiment when it was terminated.

RESULTS.

Tumours of the liver.

In the " single " rats treated with 15 doses of A.A.F. only benign lesions of the liver were seen when the animals were killed in the 52nd week of the experiment. In the group treated with 24 doses malignant hepatomas were also found from the 37th week onwards. The results obtained in the parabiotic rats are summarized in Table I.

TABLE I.—*Tumours Induced by A.A.F. in Normal Male Partner of Parabiotic Rats.*

Pair number.	Sex of partners.	Duration of experiment (weeks).	Number of doses (4 mg.) of A.A.F. given.	Liver.	Seminal vesicle.
1	♂/castr. ♂	41	38	Metastasizing hepatoma	0
2	Ditto	38	35	Benign cholangioma	Adenocarcinoma
3	,,	39	22	Ditto	0
4	,,	18	25	,,	0
5	,,	42	11	,,	0*
6	,,	42	24	,,	0
7	,,	44	24	,,	0
8	,,	51	6†	0	0
9	,,	59	24	Metastasizing hepatoma	Adenocarcinoma
10	,,	46	24	Malignant hepatoma	,,
11	,,	47	24	Ditto	0
12	,,	46	27	,,	0
13	♂/spayed ♀	46	18	Benign cholangioma	Adenocarcinoma
14	Ditto	48	18	Ditto	0
15	,,	33	18	,,	0
16	,,	43	17	,,	0
17	,,	47	20	,,	0
18	♂/♂	52	24	Hepatoma (early)	0
19	,,	52	24	Ditto	0
20	♂/castr. ♂	41	28‡	Benign cholangioma	Adenocarcinoma

* Multiple abscesses in seminal vesicle and prostate.
† Six doses of 2 mg. of A.A.F.
‡ Diet given for four weeks containing 0·03 per cent of A.A.F.

In the parabiotic rats the liver of the normal partner treated with A.A.F. was always enlarged and invariably showed neoplastic changes when doses of 4 mg. had been given. Fig. 1 and 3 illustrate the different appearance of the livers of the two partners. Rats treated with 24 or more doses and surviving for at least 41 weeks were found to have hepatomata, two of which had metastasized into the lungs of the animal bearing the hepatoma (Fig. 2). The livers of rats which had received less A.A.F. contained benign cystic cholangiomata. These were most conspicuous in the left lobe and the lobus caudatus. In the experiment where the intact partner had been treated with only 6 doses of 2 mg. of A.A.F., the liver was free of neoplastic lesions, but larger than the one of its partner. The liver of the litter-mate not treated with A.A.F. was never affected. Not a single lesion characteristic for the action of A.A.F. was seen in any of the rats joined to a partner to whom the carcinogen had been administered. These livers were macroscopically and histologically normal. In the pair of rats who had received A.A.F. in their food cystic cholangiomata were found in both.

Tumours of the seminal vesicle.

The seminal vesicles were the only other organ in which neoplastic changes have been found. These occurred only in rats which received 18 or more doses

of the carcinogen and survived for a minimum of 38 weeks. In nearly all the normal rats joined to a castrated or spayed litter-mate the accessory sex organs were larger than in normal animals of similar size. In 4 of these 20 rats treated with A.A.F. the hyperplastic seminal vesicles contained macroscopically visible nodules which were situated near the cephalad margin of the organ ; in addition a fifth neoplasm of microscopic size was found.

In all 5 instances the fundamental histological change was the invasion of the muscular coat of the seminal vesicle by atypical glandular epithelium and therefore the lesion must be considered an adenocarcinoma. At one or more points the sharp limitation of the glandular elements from the muscular coat, characteristic for the normal organ, was lost and both elements intermingled. The pattern of the glandular epithelium became distorted and the epithelium lining the cystic spaces assumed variable sizes and shapes, mitosis being moderately frequent. Fig. 4 and 5 illustrate the earliest neoplastic changes found in a seminal vesicle, which macroscopically appeared only hyperplastic. This carcinoma was still relatively free from the secondary changes observed in most of the more advanced tumours. Once the invading carcinoma cells had reached a certain distance from their point of origin they frequently lost their characteristic glandular arrangement and the tumour became anaplastic (Fig. 6). At the same time inflammatory changes became more and more prominent. Polymorph leucocytes, round cells, fibroblasts and newly formed blood vessels were found between nests of tumour cells (Fig. 7, 8), together with multinucleated giant cells (Fig. 9), some of which were probably derived from desintegrating muscle cells. Sometimes it became difficult to decide whether a lumen was lined by tumour cells or by young proliferating endothelium. However, the large vesicular nucleus of the tumour cells helped in their identification. In other areas signs of inflammation were lacking and invading carcinoma cells arranged in form of alveoli or tubules were lying enmeshed in densely fibrotic tissue, which had replaced the muscle (Fig. 10). The destruction of the muscular coat seemed to be due partly to the tumour cells and partly to the inflammation accompanying the invading neoplasm.

Fig. 11 and 12 illustrate the appearance of the only tumour of the seminal vesicle found previously in our material. This large anaplastic carcinoma arose in a seminal vesicle of an albino rat of the " Sheffield " strain which had received a diet containing A.A.F. and para-amino benzoic acid. In another case both seminal vesicles and prostates formed one large mass containing multiple cysts filled with yellowish creamy material. Adhesions connected this mass with loops of the bowel. This lesion was of inflammatory origin and no neoplastic changes were found in it although many sections were studied.

As already mentioned not a single neoplastic lesion was found in the castrates joined to brothers treated with A.A.F. In the pair where both received the carcinogen with their food, the kidney of the castrate showed a lesion of microscopic size which we have not found so far in rats treated with A.A.F. Near the surface of the kidney an area of the cortex was replaced by nests of cuboid cells with pale cytoplasm and round nuclei with well dispersed chromatin. Except for greater variation in size, these nuclei resembled closely those seen in normal tubular epithelium. The cells situated near the periphery of the lesion were highly vacuolated and some seemed to disintegrate, only the cell membrane remaining. Delicate collagen fibres surrounded the tubular structures as shown

in Fig. 14. The whole picture resembled that of a benign tubular adenoma of the kidney.

Morphological Signs of Increased Androgen Secretion in the Normal Parabiotic Male Partner.

The changes to be described were found in most cases and were independent of the administration of A.A.F. The testes were frequently increased in size. Their tubules were normal ; the Leydig cells were large, having ample cytoplasm and in some animals their numbers had increased. The breast glands were stimulated and consisted mainly of cystic dilated ducts, the lumen of which was filled with amorphous eosinophilic material. The hyperplasia of prostate and seminal vesicle has already been mentioned. In the latter the glandular part of the organ was more stimulated than the outer mesenchymal layer. This disproportionate growth led to compression of the epithelial folds lined by high cylindrical cells. The suprarenals of the intact partner differed histologically from those of the castrated twin. The glomerulosa of the former was denser and consisted mainly of cells the cytoplasm of which stained well with eosin. The fascicularis contained signet-ring cells. However, they were not as frequent as in the example illustrated by Selye (1947) on p. 133 of his text-book of endocrinology. In the castrate the glomerulosa was formed by large pale cells, the fascicularis was free of signet-ring cells and the reticularis was hyperaemic and better developed than in the normal twin. The kidneys of the normal partner were larger than the ones of the castrate. Their surface was more granular and histologically the tubular apparatus showed slight degenerative changes, the connective tissue being more prominent, especially in the outer layer of the cortex. The pituitaries of the normal litter-mates were consistently smaller than in the castrates and were sometimes even below the weights normally found in our rats of similar size. In these pituitaries the numbers of acidophils were increased with a corresponding decrease in chromophobes. The basophils were less affected. Only in one instance a large nest of basophils was found which could be interpreted as a beginning adenoma (Fig. 13). However, in this nodule of microscopic size only a few mitoses were seen and there was little compression of the surrounding tissue. A fair number of cells showed regressive changes, such as pyknosis of the nucleous and extreme vacuolation of the cytoplasm. The majority of the elements forming this structure consisted of large cells with a faintly blue cytoplasm. In sections kindly stained by Dr. W. E. Griesbach with the periodate-Feulgen technique, the cells gave a slight Schiff reaction. This agglomeration of basophils was probably not a permanent structure. Zahler (1950) has described the appearance of nests of basophils in the pituitaries of rats treated with testosterone.

The state of parabiosis did not seem to influence the growth of the rats, nor to increase their susceptibility to A.A.F. In the two pairs where two normal males had been joined together, the animals treated with 24 doses of A.A.F. reached the same size as " single " rats, which had received the same dosage of the carcinogen. Growth inhibition, however, did occur in most instances where a normal male had been joined to a castrated or spayed litter-mate. Table II gives the body weight of the two partners as well as the weights of liver, pituitary and testes.

TABLE II.—*Body Weights and Weights of Livers and Pituitaries of both Partners and of Testis of Normal Litter-mate.*

Pair number.	Body weights (g.).		Liver (g.).		Pituitary (mg.).		Testis (g.).
	A.	B.	A.	B.	A.	B.	A.
1	189	218	28·7	9·4	4·2	9·4	—
2	140	179	516	6·6	3·8	8·5	1·97
3	148	162	6·7	6·5	4·3	9·8	—
4	120	167	6·8	6·7	—	—	—
5	130	182	16·4	8·2	2·9	9·7	—
6	225	285	8·0	10·0	6·1	11·3	3·86
7	286	271	14·0	9·0	7·9	10·6	4·43
8	305	323	12·4	11·2	9·5	14·3	—
9	277	263	18·4	10·0	7·4	10·2	3·19
10	321	278	36·7	10·4	6·8	12·8	4·1
11	224	294	11·2	10·0	6·2	11·5	3·7
12	278	271	18·8	—	6·8	11·0	4·2
13	312	239	14·0	10·5	7·1	21·4	2·2
14	334	274	17·5	8·5	7·6	10·0	2·73
15	258	238	19·8	7·2	8·4	10·6	2·9
16	290	282	13·9	9·2	7·0	10·8	3·04
17	298	254	13·9	9·1	8·4	10·1	3·2
18	374	376	19·5	12·0	7·3	7·6*	3·5
19	347	316	15·4	11·5	8·2	8·1*	3·6
20	267	211	9·5	8·1	7·6	11·6	3·12

A = Normal; B = gonadectomized partner.
* Not castrated.

DISCUSSION.

From the results described in this paper it is obvious that A.A.F. given by stomach tube to one partner of a pair of parabiotic rats does not affect the other, at least not when such amounts as used in this investigation are administered. In this respect the carcinogen behaves like the steroid hormones which pass only from one to the other partner when extremely high concentrations are reached in the circulation of one of them (Zeckwer, 1946).

The liver tumours obtained appeared after approximately the same interval as in " single " rats treated with similar amounts of A.A.F. Metastases occurred in two instances, proving the malignant character of these hepatomas. Although the hepatomatous liver was situated in the same peritoneal cavity as the liver of the untreated partner, the latter remained perfectly normal. There was not the slightest indication for an agent derived from the malignant hepatoma affecting the liver of the litter-mate.

As mentioned in the introduction a constant flow of gonadotropins passes from the castrate to the normal partner stimulating its gonads. However, no tumours of the testicles have been found. In this respect our experiments failed where those of Biskind and Biskind (1945) and of Twombly, Meisel and Stout (1949) succeeded. These authors obtained tumours in testes which had been grafted into the spleen. Twombly, Meisel and Stout (1949) worked with Wistar rats, the same strain as used in our investigation. The duration of their experiments was only slightly longer. It seems, therefore, that our failure to obtain tumours of the testis is due neither to incompetence of the strain nor to the time factor. The hormonal stimulus acting on a testis grafted into the spleen of a castrate is not the same as the one to which the gonads of a normal rat joined to a castrated litter-mate are exposed. Whereas the transplanted testis is stimulated by a

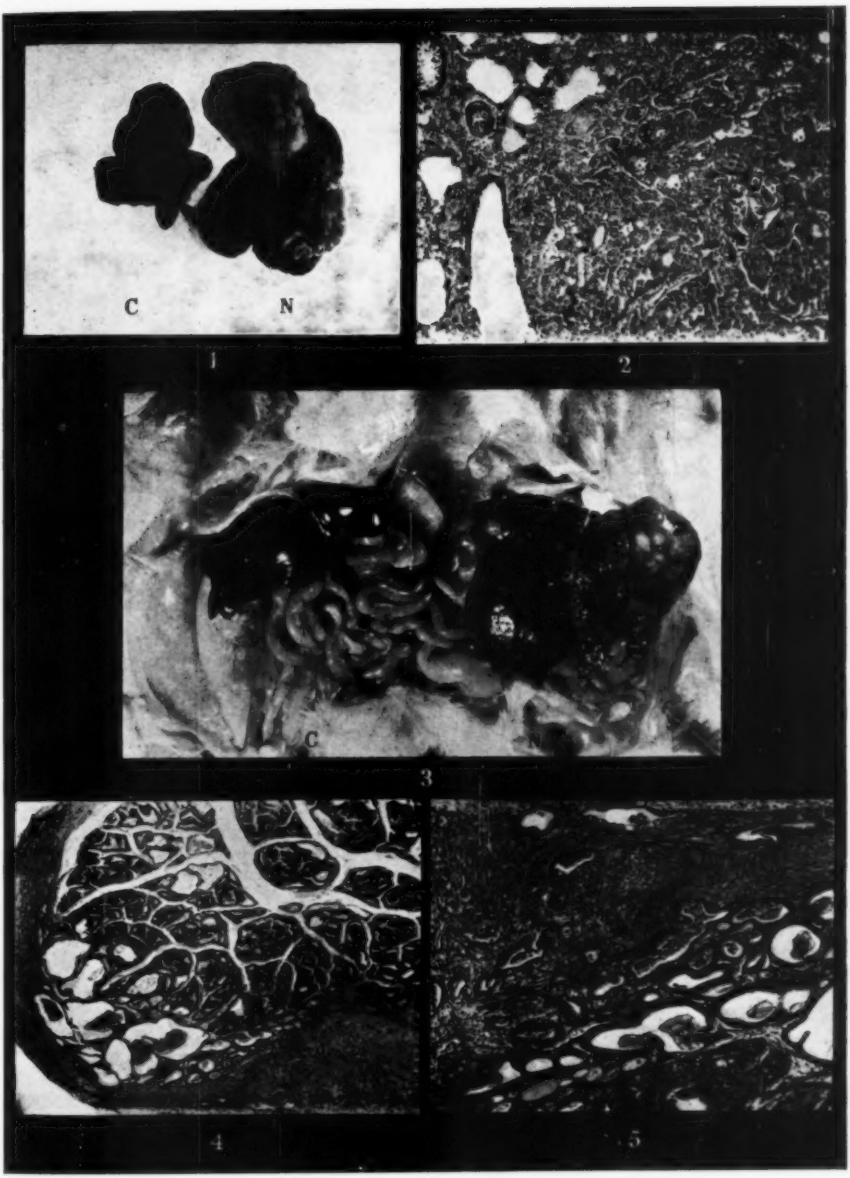
pituitary of the castrate type which secretes high amounts of F.S.H and little L.H., the testes of the parabiotic rat are under the influence of the hormones of the animal's own pituitary and of the gonadatropins coming from the castrated partner. The latter secretes mainly F.S.H., the former probably predominantly L.H., as indicated by its acidophilia. Two further points should be considered. Spermatogenesis is inhibited in testes situated in the abdomen and consequently the morphology of such gonads is considerably altered; in addition transplantation of a testis into the spleen involves traumatic injury. These two factors are not present in the experimental procedure used by us. Further studies seem to be required to elucidate the conditions essential for tumour induction in the male gonad, a problem which has been recently reviewed by Lipschutz (1950).

The androgens secreted by a testis transplanted into the spleen do not reach the general circulation and therefore the accessory sex organs of rats bearing such grafts are not stimulated. The normal parabiotic male joined to a gonadectomized litter-mate shows the effects of increased androgen secretion. In the pairs not treated with A.A.F. this stimulation did not lead to tumour formation in any of the target organs, but in the group treated with the carcinogen five tumours appeared in the seminal vesicles of normal partners. We consider this result to be significant. We have never found this type of tumour occurring spontaneously in the New Zealand strain, nor have we seen it in "single" rats treated with A.A.F. Only once a carcinoma of the seminal vesicle was observed in an albino rat of the "Sheffield" strain treated with A.A.F. Our combined material represents the post-mortem findings of nearly a thousand male rats treated with A.A.F. In the rat spontaneous tumours of the seminal vesicle are extremely rare and we have found in the literature only the case reported by Flexner and Jobling (1907). In men, tumours of this organ are also of very infrequent occurrence. Lazarus (1946) has critically reviewed the cases reported, and since then only a few additional observations have been recorded (McCree, 1948; Gee, 1948).

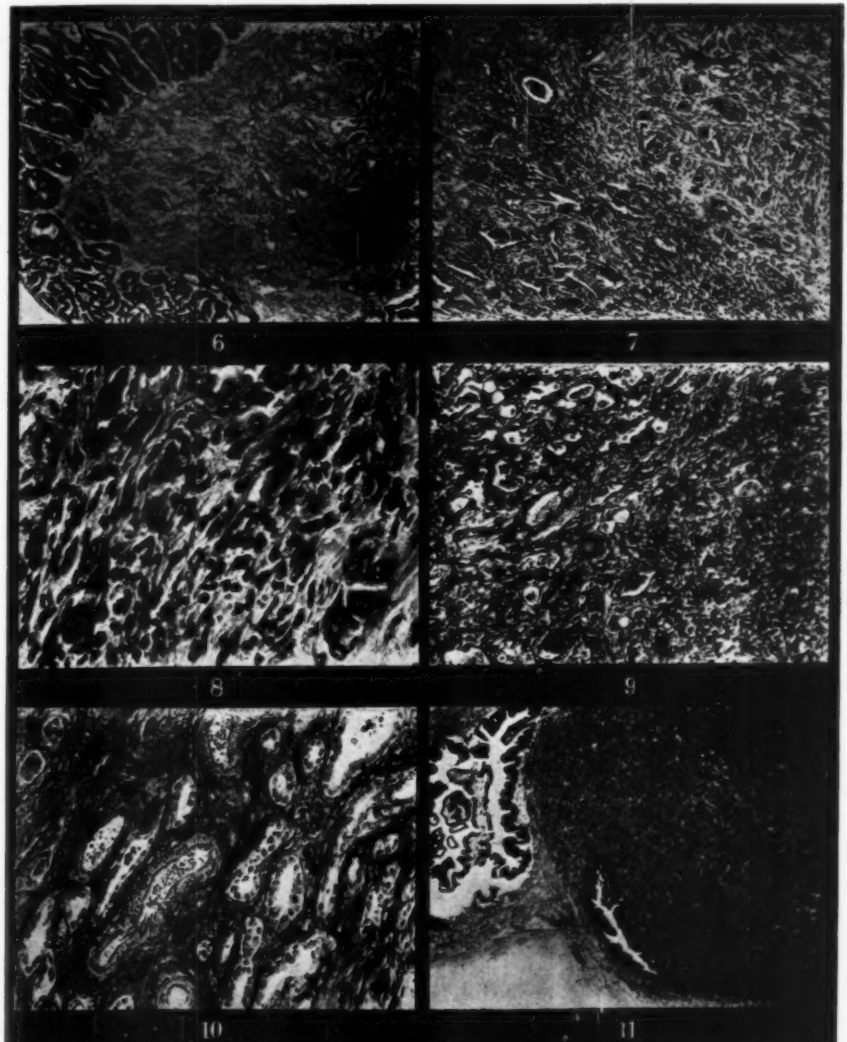
In the case of the thyroid, the carcinogenic action of A.A.F. on this organ is considerably increased by hormonal stimulation (Bielschowsky, 1944, 1945). This has been confirmed by Paschkis, Cantarow and Stasney (1948), Hall (1948) and

EXPLANATION OF PLATES.

FIG. 1.—Aspect of livers of Pair 1. N = Normal; C = castrated partner. × 0·4.
FIG. 2.—Lung metastasis of hepatoma shown in Fig. 1. × 45.
FIG. 3.—Aspect of liver of Pair 9 *in situ*. N = Normal; C = castrated partner.
FIG. 4.—Early adenocarcinoma found in seminal vesicle of normal partner (Pair 13). × 15.
FIG. 5.—Detail from Fig. 4. × 45.
FIG. 6.—More advanced carcinoma found in seminal vesicle of normal partner (Pair 11). × 25.
FIG. 7.—Another area of the tumour depicted in Fig. 6 showing destruction of muscular coat. × 45.
FIG. 8.—Detail from Fig. 7. × 300.
FIG. 9.—Area of tumour found in seminal vesicle (Pair 2) showing pronounced inflammatory changes and a giant cell near the centre. × 80.
FIG. 10.—Adenocarcinoma of the seminal vesicle. Tubular structures surrounded by coarse collagen fibres. × 80.
FIG. 11.—Anaplastic carcinoma of the seminal vesicle found in a "single" rat of the Sheffield strain treated with A.A.F. × 15.
FIG. 12.—Detail from Fig. 11. × 300.
FIG. 13.—Large nest of basophils found in pituitary of the intact partner (Pair 17). × 300.
FIG. 14.—Lesion found in kidney of the castrated partner of Pair 20. × 45.
FIG. 15.—Aspect of accessory sex organs, × 1·2.
FIG. 16.—Area of adenocarcinoma from nodule found in adhesion. × 45.
FIG. 17.—Metastasis attached to spermatic cord. × 25.



Bielschowsky and Hall.



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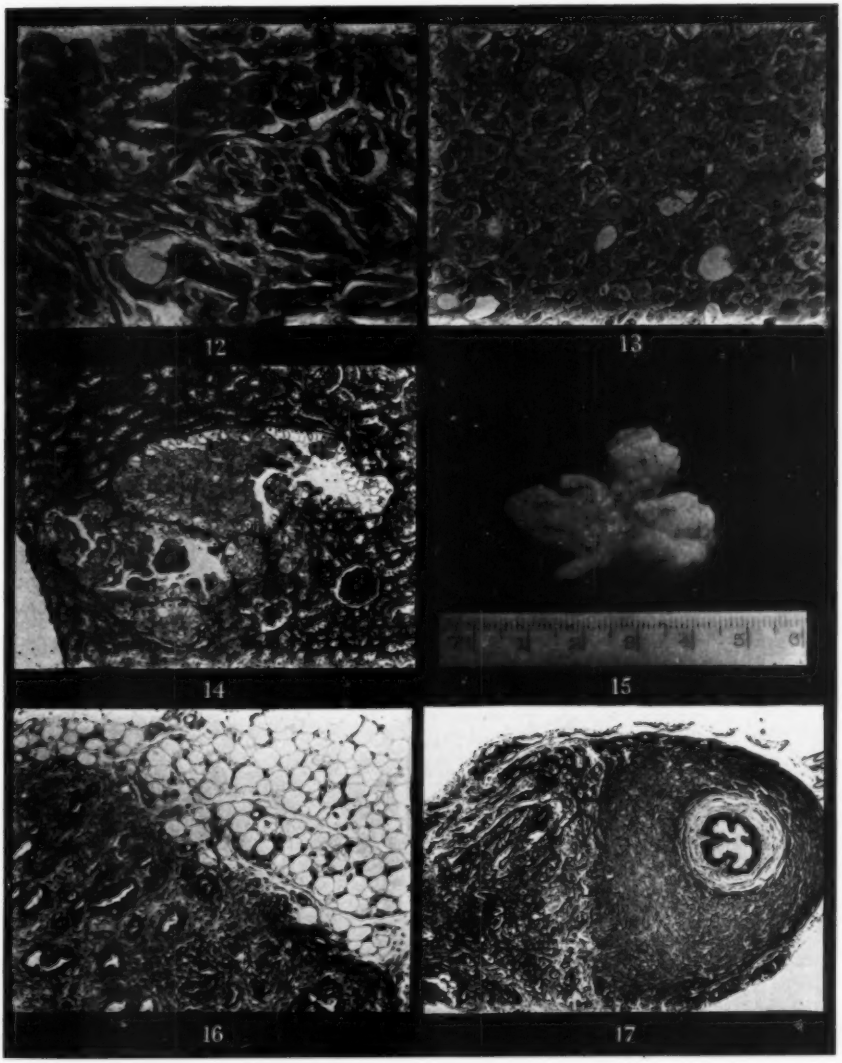

12 13

14 15

16 17

Bielschowsky and Hall.

by Doniach (1950). The same seems to hold good in the case of the seminal vesicle. The hyperplasia induced by increased amounts of androgens secreted by the stimulated testis enhances the susceptibility of the seminal vesicle to the carcinogenic action of A.A.F. The fact that tumours of macroscopic size were found only in rats treated with 24 or more doses of 4 mg. of A.A.F. and not in animals receiving less suggests that considerably more of the carcinogen is required to bring about tumour development in the stimulated seminal vesicle than was needed for the induction of adenomata in the hyperplastic thyroid (Hall, 1948).

That only one minute tumour of the seminal vesicle was found in an intact male joined to a spayed female is probably due to the lower dosage of A.A.F. given to this group and not to the nature of the gonadotropins of the spayed female. Only 17 to 20 doses of the carcinogen were administered because great difficulties were encountered in keeping alive pairs of opposite sex for periods necessary for tumour development. However, our material is not large enough to decide this question.

To summarize, we consider the tumours of the seminal vesicle to be due to a hormonal imbalance created by gonadotropins in the normal partner. These hormones, by stimulating the gonads of the normal partner, increase the androgen production of its testes and therefore are responsible for the hyperplasia of the seminal vesicles. Although unable to promote tumour growth in the testes they bring about conditions in which an accessory sex organ becomes susceptible to the action of A.A.F. The possibility cannot be excluded that still more prolonged androgenic stimulation may ultimately bring about tumour formation in the seminal vesicle without the aid of A.A.F.

ADDENDUM (received for publication February 7, 1951).

After the above paper had been submitted an additional pair of parabiotic rats had to be sacrificed. In this pair a normal male was joined in parabiosis to a castrate, the former having received 20 doses of A.A.F. by stomach tube. In the 36th week of the experiment the health of the normal partner declined rapidly. Besides an enlarged liver two hard nodules of approximately the size of a cherry could be palpated in the lower part of the abdomen. The post-mortem examination confirmed the presence of a greatly enlarged liver containing multiple benign cystic cholangiomata. The abdominal nodules were, in fact, the two seminal vesicles and coagulating glands which were found transformed into irregular masses (Fig. 15). They were white in colour, hard, and solid throughout. When cut, the lumen of the glands was not recognizable. Histological examination revealed that most of the normal tissue had been destroyed by an adenocarcinoma which was of similar structure to the smaller neoplasms of the seminal vesicle previously described. Adhesions connected the left seminal vesicle with loops of small intestine and a hard small nodule was found embedded in these adhesions at approximately 3 mm. distance from the upper margin of the left tumorous gland. Fig. 16 shows the microscopic appearance of the lesion. Histologically it consisted of acini lined by atypical epithelium surrounded by a fairly cellular connective tissue containing a few collagen fibres. Comparison with the neoplastic seminal vesicles showed close resemblance in structure, but it could not be decided whether the nodule represented a blood or lymph borne metastasis or whether this deposit of tumour tissue was the result of continued

growth of the primary tumour into the loose tissue of the adhesion. However, a true metastasis was found at another site. Attached to the posterior and lateral surfaces of the left spermatic cord above the point where it was severed a small nodule was present, just recognizable in Fig. 15. It proved to be an area of adenocarcinoma which had invaded the outer muscular layer of the spermatic cord (Fig. 17). It had the same structure as the primary tumour and the nodule present in the adhesion.

This case proves that the neoplastic lesions found in seminal vesicles of normal male rats treated with A.A.F. and joined in parabiosis to a castrate can progress to the state of true malignancy.

SUMMARY.

(1) The tumours induced by A.A.F. in normal male rats joined in parabiosis to castrated or spayed litter-mates have been described. Neoplasms were only obtained in animals receiving the carcinogen and never in their untreated litter-mates.

(2) Benign or malignant tumours of the liver were found depending on the amounts of the carcinogen given and on the duration of the experiment. The malignant hepatomas metastasized to the lungs of the animal bearing the cancer of the liver, but never " infected " the other partner.

(3) Adenocarcinomata of the seminal vesicles were found in 5 rats. These neoplasms are considered to be due to two factors; the carcinogenic action of A.A.F. and the hyperplasia induced in the seminal vesicle by high levels of androgens.

A further example of adenocarcinoma of the seminal vesicle is described in an addendum. The tumour was larger than those previously encountered and had metastasized.

REFERENCES.

Bielschowsky, F.—(1944) *Brit. J. exp. Path.*, **25**, 90.—(1945) *Ibid.*, **26**, 270.
Biskind, M. S., and Biskind, G. R.—(1944) *Proc. Soc. exp. Biol. N.Y.*, **55**, 176.—(1945) *Ibid.*, **59**, 4.
Doniach, J.—(1950) *Brit. J. Cancer*, **4**, 223.
Flexner, S., and Jobling, J. W.—(1907) *Zbl. allg. Path. path. Anat.*, **17**, 257.
Gee, E. M.—(1948) *Brit. J. Urol.*, **20**, 72.
Hall, W. H.—(1948) *Brit. J. Cancer*, **2**, 273.
Jacobsohn, D.—(1948) *Acta physiol. scand.*, **17**, suppl., 57.
Lazarus, J. A.—(1946) *J. Urol.*, **55**, 190.
Lipschutz, A.—(1950) 'Steroid Hormones and Tumours.' Baltimore (Williams & Wilkins Co.).
Martins, Th.—(1929) *C. R. Soc. Biol., Paris*, **103**, 1341.
McCree, L. E.—(1948) *J. Amer. med. Ass.*, **136**, 679.
Paschkis, K. E., Cantarow, A., and Stasney, J.—(1948) *Cancer Res.*, **8**, 257.
Selye, H.—(1947) 'Textbook of Endocrinology.' Univ. de Montreal (*Acta Endocrinologica*).
Strömbeck, J. P., and Ekman, B.—(1949) *Acta path. microbiol. scand.*, **26**, 480.
Twombly, G. H., Meisel, D., and Stout, A. P.—(1949) *Cancer*, **2**, 884.
Zahler, H.—(1950) *Virchows Arch.*, **317**, 547.
Zeckwer, I. T.—(1946) *Endocrinology*, **38**, 249.

FURTHER OBSERVATIONS ON THE LIVER CATALASE DEPRESSING ACTION OF TUMOURS.

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RECENT work has provided evidence in favour of the supposition that tumours contain, and therefore probably release into circulation, a substance capable of depressing liver catalase activity. Nakahara and Fukuoka (1949) claimed to have produced a liver catalase depression in mice after the injection of alcohol-precipitated fractions obtained from human tumours. Similar fractions from normal tissue were stated to be inactive. Adams (1950*a*) commenced a study of the effect of the injection of homogenized mouse tumours into mice, and showed that after an early (24 and 48 hours) depression, the liver catalase level returned to normal approximately 4 days after injection, and fell once more as the new tumour grew. The results were interpreted as indicating that the initial depression is due to some substance or substances present in the injected material, the second depression only being due to the new tumour. Injection of homogenized normal tissues had no effect on catalase level.

Appleman, Skavinski and Stein (1950), in the course of an investigation of the variations in erythrocyte, kidney, and liver catalase activity in rats bearing the Jensen sarcoma or the U.C.L.A. fibrosarcoma, found that normal rats kept on a protein-free diet showed a drop in liver catalase activity to approximately 50 per cent of normal within 7 to 10 days from the time they were placed on the diet, and this low level was maintained for 53 days or longer. Two to three days after these animals had returned to a normal diet the liver catalase activity returned to normal. These authors consider their results in harmony with the alternative hypothesis, that tumours abstract from circulation some essential component required for the maintenance of a normal catalase level. However, the results already obtained in this laboratory, and those about to be presented, showing that a reduction of liver catalase level can readily be obtained in the absence of tumour growth, give definite evidence for the activity of some toxic material present in tumour tissue.

MATERIALS AND METHODS.

Animals.

The greater part of this work has been carried out using young adult mice (weighing 25 to 30 g.) of a stock albino strain. Two other strains have been used for individual experiments—FF fawn mice (Glaxo Laboratories) and albino mice obtained from the National Institute for Medical Research, Hampstead, known as the "Parkes" strain. During the period of the experiment the diet of the animals consisted of rat cubes and water *ad libitum*. As a routine the mice were starved for 12 hours before catalase determination.

Tumours.

(a) *Mouse.*—Sarcoma 37, Sarcoma 2146, Crocker sarcoma 180, Leukaemias FAK 1 and FAK 2, C57 sarcoma and Adenocarcinoma A, Strong A mammary carcinoma, CBA slow-growing sarcoma (kindly supplied by the Chester Beatty Research Institute, and the Imperial Cancer Research Fund Laboratories).

(b) *Rat.*—RIB 1 (slow-growing) and RIB 5 (fast-growing) sarcomata, originally produced in pure line albino rats by subcutaneous injection of a carcinogen, and kindly supplied by Dr. R. John (both tumours after *circa* 100 passages); Jensen rat sarcoma; primary hepatoma produced by feeding aminoazobenzene, kindly provided by Mrs. C. Hoch-Ligeti.

(c) *Human.*—Oat-cell carcinoma of lung (kindly provided by Dr. Russel Brain); rectal carcinoma and breast carcinoma (kindly provided by Mr. D. V. Evans, F.R.C.S.). Fresh samples were taken from material removed at operation.

Tumour material was administered by the injection of a coarse suspension prepared with a TenBroeck grinder, except where otherwise stated.

Liver catalase estimation.

Catalase has been estimated by allowing liver homogenates to react with M/40 hydrogen peroxide in M/50 phosphate buffer (pH 6·8) at 0° C. as previously described (Adams, 1950*a*).

RESULTS.

Tumours which grow in the strains of mice used.

The results already obtained had shown that in two strains of mice the injection of homogenized S37 tissue resulted in a marked depression in liver catalase activity 24 and 48 hours later, a rise to normal at about 4 days, and a further progressive fall down to about 30 per cent of normal with the growth of the new tumour (Adams, 1950*a*). This work has been repeated, using Sarcoma 2146 and the Crocker sarcoma 180. The variations in liver catalase level following the subcutaneous injection of coarse suspensions of these tumours into albino mice appear in Table I. Results in both males and females were similar to those previously obtained with S37 and Carcinoma 63 (Adams, 1950*a*), i.e., an early depression followed by a rise towards normal, and a subsequent fall with the growth of the new tumours. In no case was tumour growth observed before 5 to 6 days after injection.

No results are recorded after 7 days for the stock albinos injected with S2146, since tumours only appeared in approximately half the animals.

As previously observed with S37 and Carcinoma 63, the males appear more sensitive than the females. In fact, with S2146 and Parkes albinos there was little, if any, lowering of catalase level in the females. This differential effect has now been consistently observed with 4 tumours and 3 strains of mice.

Effect of injection of disintegrated or partly centrifuged S37 sarcoma.

It seemed of interest to attempt to produce a catalase depression in the complete absence of tumour growth. This would show beyond doubt that the depression must be due to some material contained in the original tumour tissue.

TABLE I.—*Effect on Liver Catalase of Sarcoma 2146 and the Coarse Homogenates* of 1. Doses.*

TABLE I.—*Effect on Liver Catalase*

Results are
Each treate

Strain.	Tumour.	Contro
Stock ♂	S2146	169 ±
Stock ♀		131 ±
Parkes ♂		124 ±
Parkes ♀		75 ±
Stock ♂	Crocker sarcoma 180	175 ±
Stock ♀		122 ±

* "Coarse homogenates" were those whi
Θ = Not significant.

TABLE II.—*Effect on Liver Catalase o*
and Pure Line

Each treate
Results are

Tissue.	Route.			
FAK I	S			
FAK II	I			
C57 sarcoma	S			
C57 adenocarcinoma A	S			
CBA slow-growing	I			
Strong A mammary	S	—	—	
Rat tumours.				
Jensen rat sarcoma	S	157 ± 6	176 ± 13	154 ± 12
Hepatoma	I	143 ± 8	144 ± 15	156 ± 13
RIB I	S	136 ± 11	148 ± 14	141 ± 11
RIB 5	I	120 ± 10	113 ± 8	118 ± 5
Human tumours.				
Rectal carcinoma	I	120 ± 10	112 ± 11	115 ± 9
Oat-cell lung carcinoma	I	121 ± 10	112 ± 11	120 ± 7
Breast carcinoma	S	141 ± 8	144 ± 11	155 ± 7

Θ = Not significant.

ERRATUM

Adams, D.H., p. 117, Table 1:

Under "4 days" read "150±9" for "60±9".

Coarse Homogenates of Sarcoma 2146 and the*
. Doses.

lard error of means.
ach control group eight.

nits/mg. N.			Per cent 48-hour depression.	Significance of 24-and 48-hr. depression.	
7 days.	8 days.	10 days.		t (24-hr.).	t (48-hr.)
158 ± 10	—	—	31	4·0	4·3
130 ± 10	—	—	17	Θ	2·1
126 ± 14	—	95 ± 8	35	2·9	3·2
79 ± 8	—	68 ± 7	10	Θ	Θ
—	110 ± 6	—	27	Θ	3·4
—	101 ± 7	—	20	Θ	2·2

ıd therefore, presumably, contained intact tumour cells.

ritoneal (I) of Coarse Homogenates of Rat, Human
Male Mice. 100-*mg. Doses.*

ach control group eight.
dard error of means.

units/mg. N.		Significance of 24 and 48 hour depression.	
4 days.	7 days.	t(24-hr.).	t(48-hr.).
109 ± 13	142 ± 10	Θ	3·8
134 ± 12	—	2·6	3·4
139 ± 8	—	Θ	2·3
121 ± 7	142 ± 11	Θ	2·8
146 ± 9	—	Θ	2·9
152 ± 7	—	2·5	4·1
162 ± 6	—	Θ	Θ
138 ± 12	—	Θ	Θ
142 ± 9	—	Θ	Θ
120 ± 5	—	Θ	Θ
131 ± 9	—	Θ	Θ
128 ± 8	—	Θ	Θ
143 ± 7	—	Θ	Θ

Two general methods have been employed: the first involved the injection of S37 sarcoma, which had been treated so as to render it incapable of giving rise to tumour growth, and the second, the results of which are presented in the next section, the injection of pure line mouse tumours which do not grow in the strains of mice used.

Sarcoma 37 tissue was disintegrated by shaking for several minutes with glass beads in a "Mickle" tissue disintegrator, made by Messrs. H. Mickle, Romeyn Road, Streatham, London. In this machine a specially shaped vessel vibrates with an amplitude of about 1 cm., 50 times per second. The material was then

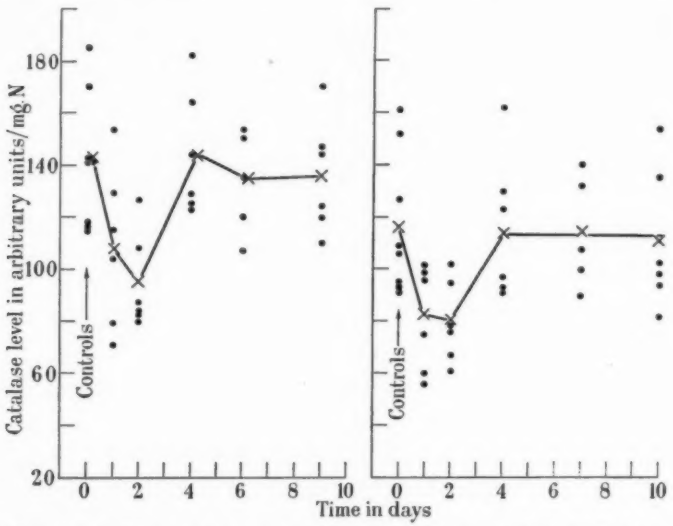

FIG. 1.—Variation in liver catalase level after the subcutaneous injection of disintegrated Sarcoma 37 tissue into FF male mice (70 mg. per mouse). Points represent levels in individual mice, and the crosses the arithmetic mean values of the groups, in this and the following figures. Left-hand graph: Tissue disintegrated in normal saline. (Tumours appeared in 3/6 mice 12 days after injection.) Right-hand graph: Tissue disintegrated in M/100 citric acid. (No tumours grew.)
Ordinates: Catalase level in arbitrary units/mg. N.
Abscissa: Time in days.

injected into FF male mice. A stained film of the inoculum showed no intact cells. Fig. 1 is a plot of the subsequent variation in liver catalase level over a period of 10 days, the crosses representing the arithmetic mean values of the groups.

In the experiment represented in the left-hand graph the tissue was disintegrated in normal saline (pH 6·8), and it will be seen that a 24-and 48-hour depression was obtained, the level rising to normal at 4 days and remaining so at 7 and 10 days. However, this treatment was not completely successful in preventing tumour growth, in spite of failure to discover intact cells in the homogenate microscopically. Tumours appeared 12 days after injection in 3/6 mice set aside for observation. This was presumably due to the presence of a few

intact cells in the injected material. The experiment was repeated, using M/100 citric acid as the suspending fluid (pH 5·5), and the results appear in the right-hand graph. The catalase depressing effect is very similar, but in this case no tumours appeared in 18 mice kept for 3 months, when the experiment was terminated.

A second method involved the partial centrifugation of a fine homogenate of S37 tissue in normal saline, prepared originally with a TenBroeck grinder. The suspension was centrifuged until approximately one-third of the suspended material (including all or nearly all the remaining intact cells) had sedimented. The cloudy supernatant was carefully removed, and injected into FF male mice, in amounts corresponding to approximately 70 mg. of original tissue per mouse. As shown in Fig. 2 a catalase depression was observed, which was somewhat smaller than that normally produced by injection of the same quantity of uncentrifuged material. Again, no tumours were observed in 6 mice kept for 3 months.

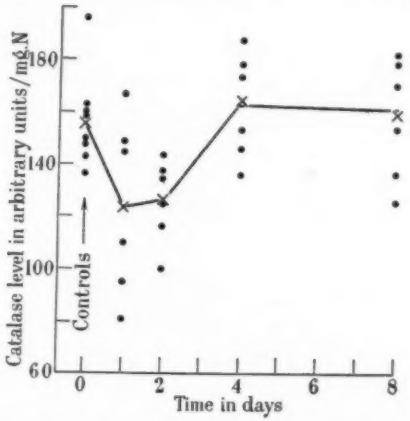

FIG. 2.—Variation in liver catalase level after the subcutaneous injection of partly centrifuged coarse homogenate of Sarcoma 37 tissue into stock albino male mice (dose equivalent to 70 mg. original tissue per mouse).
Ordinate: Catalase level in arbitrary units/mg. N.
Abscissa: Time in days.

Effect of injection of tumours which do not grow in the strain of mice used.

A number of pure line mouse tumours which do not grow, at any rate after the injection of coarse homogenates, in the stock albinos used, were injected subcutaneously or intraperitoneally in 100 mg. doses, and the liver catalase level determined at intervals. The results appear in Table II. In every case a significant depression in catalase activity was observed 48 hours subsequently, with a return to normal by the 4th to 7th day.

In one experiment of this series, not recorded in Table II, a different result was obtained. When C57 sarcoma was first used it must have become bacterially infected, since abscesses developed rapidly at the sites of injection. The catalase level at 48 hours was very low, and remained low at 4 days; at 10 days, of the 6 remaining mice, 3 in which the abscesses were healed had high levels and 3

in which they were still discharging pus had low levels of catalase activity. In Fig. 3 the results of this experiment are compared with those of a subsequent one, in which non-infected C57 sarcoma was used. The depression produced by the infected material is much greater and more prolonged.

A number of rat and human tumours were homogenized and injected into mice. The results are given in Table II. No significant variations in catalase level were observed, and with the rat tumours in particular the variations appear to be completely random.

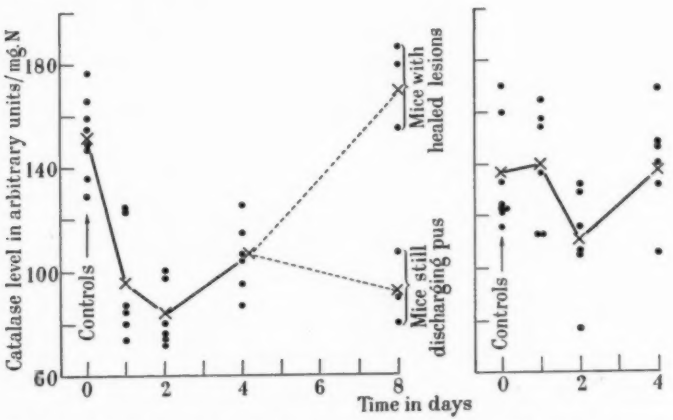

FIG. 3.—Variations in liver catalase level after the subcutaneous injection of coarse homogenate of C57 sarcoma into stock albino male mice (100 mg. per mouse). Left-hand graph: Accidentally infected tissue (causing abscess formation in under 24 hours). Right-hand graph: A subsequent experiment with non-infected tissue.
Ordinates: Catalase level in arbitrary units/mg. N.
Abscissa: Time in days.

In Fig. 4 catalase level (48 hours after intraperitoneal injection) is plotted against S37 tumour dosage. The points lie on a slightly curved line, showing a progressive decrease of catalase activity with dose.

The depression is seen to be approximately proportional to dosage over the range 10 to 30 per cent. Assuming that this holds for other tumours, a comparison may be made between the magnitude of the depressions given by those mouse tumours which grow, and those which do not, by correcting for variations dose.

The results of such a comparison appear in Table III, in which catalase depressions at 48 hours produced by different tumours are given, corrected to a 50-mg. in dose.

They indicate that the reduction in liver catalase activity produced by pure line mouse tumours which do not grow in the strain used lies between that produced by rat and human tumours (which had no effect) on the one hand, and by mouse tumours which do grow, on the other. The average depression of catalase 48 hours after injection given by the non-growing mouse tumours is approximately half of that given by the tumours which are capable of growth.

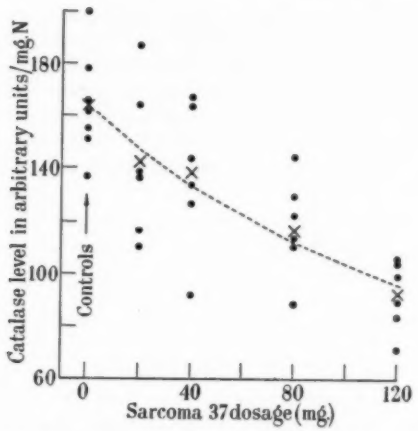

FIG. 4.—Liver catalase levels 48 hours after the intraperitoneal injection of varying quantities of coarse homogenate of Sarcoma 37 into stock albino male mice.
Ordinate: Catalase level in arbitrary units/mg. N.
Abscissa: Tumour dosage in mg.

TABLE III.—*Mouse Tumours. 48-hour Depression of Liver Catalase Calculated on Arithmetic Mean Values, and Corrected to a 50-mg. Dose.*

Tumours which grow.		Tumours which do not grow.	
Tumour.	Per cent depression at 48 hours.	Tumour.	Per cent depression at 48 hours.
S37	23–35	FAK I	16
M63	24	FAK II	11
S2146	31	C57 sarcoma	10
	(stock albinos)	C57 adenocarcinoma A	15
	35	CBA slow growing	10
	(Parkes albinos)	Strong A mammary	17
Crocker sarcoma 180	27		

DISCUSSION.

The evidence so far presented in this and the previous paper (Adams, 1950*a*) in favour of the hypothesis that tumours contain a toxic substance capable of depressing liver catalase activity may be summarized as follows:

(1) Subcutaneous injection of coarse homogenates of 4 different tumours into male mice resulted in an early depression and rise to normal before signs of tumour growth appeared. Female mice showed a similar, though less pronounced, change.

(2) Subcutaneous injection of S37 tissue thoroughly disintegrated by shaking with glass beads in M/100 citric acid pH 5·5, and of a partially centrifuged S37 homogenate, resulted in depression of catalase activity, but no tumour growth.

(3) Subcutaneous or intraperitoneal injection of 6 pure line mouse tumours which do not grow in the stock albino strain used gave an early depression, followed by a rise to normal within 4 to 7 days.

It is not claimed, however, that nutritive effects play no part in producing the depression of catalase activity, particularly in animals bearing large tumours. Miller (1950) has recently shown that although catalase level in animals which are maintained on low or non-protein diets parallels approximately the level of total liver protein, the enzyme soon begins to disappear more rapidly than the remaining protein. Consequently, even if the only relevant effect of a large tumour on the nutritional status of an animal were a simple depletion of protein, a depression of catalase would result. However, the depression which occurs in the early stages of tumour growth may be expected to be due mainly to toxic material produced by the tumour, since the importance of purely nutritive factors, e.g., protein depletion, must be small at this stage, though becoming progressively greater as the tumour size increases. The effect of large tumours on catalase is probably the resultant of separate nutritive and toxic actions, which could not be readily distinguished.

The effect of injecting infected C57 sarcoma is quoted as a possible source of error in investigations of this nature. It is not yet known whether catalase depression is a common result of bacterial infections, but this possibility must be reckoned with when using, for example, human intestinal tumours, which are often infected. Greenstein (1947) states that infection of mice with certain strains of poliomyelitis virus, or with streptococci and staphylococci, has no effect on catalase activity. However, it has been observed in this laboratory during epidemics of a salmonella infection, and of ectromelia, that catalase level was low when the liver showed signs of involvement. Dounce and Shanewise (1950) have recently shown that catalase activity is significantly depressed in rats suffering from advanced murine leprosy. Apart from these isolated observations, no detailed investigation has been made of the effect on liver catalase of infections of the liver, or of injections of infected normal or malignant tissue.

Although a depression of liver catalase may occur in conditions other than cancer, this does not detract from the importance of the effect as a property of malignant but not of normal tissue, and work on the mechanism by which the toxic substance exerts its effect may throw light on some fundamental difference between normal and malignant tissue. Recently reports of preliminary experiments have appeared, indicating the importance of hormones in maintaining a normal level of liver catalase (Begg and Reynolds, 1950; Adams, 1950*b*). The present investigation is being followed up by an endeavour to relate the tumour-depressing effect with the hormonal control mechanism.

Depression of liver catalase activity has been shown to follow the injection into mice of mouse tumours, but not of rat and human tumours in single doses of up to 100 mg. Nakahara and Fukuoka (1949), on the other hand, observed a depression after the injection of alcohol-precipitated fractions of human tumours into mice. However, their dosage, referred to weight of original tissue, was probably very much higher than that used here. All that is now claimed is that injection, in the dose used, of mouse tumours into mice produces a considerable depression of liver catalase, while injection of similar doses of tumours of other species produces no apparent change. The precise significance of the observed specificity is not clear. The fact that mouse tumours which do not grow give effects intermediate between those of mouse tumours which grow and tumours of other species, suggests that the capacity of a tumour for growth in a particular host may be connected with its catalase depressing activity. Greenstein and

Ancervont (1942) have already stated that, in general, tumours which grow rapidly give larger catalase depressions than those which do not. Possibly the catalase depressing substance exercises some protective effect on the tumour itself. At any rate, since the catalase depressing substance is capable, in relatively small dosage, of affecting so greatly at least one liver enzyme, and probably others, its influence on the enzyme pattern in the tumour cells, where its concentration must be far higher than in the circulation, may well be profound.

SUMMARY.

(1) Injection of coarse homogenates of Sarcoma 2146 and Crocker sarcoma 180 in 50-mg. doses gave significant depressions of catalase level 48 hours after injection followed by a rise to normal at 4 days, as had previously been observed with Sarcoma 37. No tumour growth was observed earlier than 6 days after injection. Males were more sensitive than females. The early variation in females was not significant in one case.

(2) Injection into stock albino mice of Sarcoma 37 tissue (*a*) disintegrated in M/100 citric acid, or (*b*) partly centrifuged after coarse homogenization in 70 mg. doses, resulted in a depression of liver catalase without any observable tumour growth.

(3) Six pure line mouse tumours, which do not grow in the stock mice used, also gave catalase depressions 48 hours after injection in 100-mg. doses, followed by a return to normal at 4 or 7 days.

(4) Rat and human tumours in 100-mg. doses had no effect on mouse liver catalase.

My thanks are due to Professor S. P. Bedson for his interest, and to Dr. M. H. Salaman for his suggestions and advice. I am also indebted to Mr. L. J. Hale for skilled technical assistance, and to Mr. G. Downes for his care of the animals. Acknowledgments for the supply of tumour material are given in the text. I would, however, like to thank Mr. J. Marsh, of the Chester Beatty Research Institute, and Miss Griffiths, of the Imperial Cancer Research Fund Laboratories, for their helpfulness in providing a number of mouse tumours.

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REFERENCES.

Adams, D. H.—(1950*a*) *Brit. J. Cancer*, **4**, 183.—(1950*b*) *Nature*, **166**, 952.
Appleman, D., Skavinski, E. R., and Stein, A. M.—(1950) *Cancer Res.*, **10**, 498.
Begg, R. W., and Reynolds, E. F.—(1950) *Science*, **111**, 721.
Dounce, A. L., and Shanewise, R. P.—(1950) *Cancer Res.*, **10**, 103.
Greenstein, J. P.—(1947) 'Biochemistry of Cancer.' New York (Academic Press).
Idem and Andervont, H. B.—(1942) *J. nat. Cancer Inst.*, **2**, 345.
Miller, L. L.—(1950) *J. Biol. Chem.*, **186**, 253.
Nakahara, W., and Fukuoka, F.—(1949) *Gann*, **40**, 45.

THE ACTION OF SOME PYRIMIDINE DERIVATIVES AND RELATED COMPOUNDS ON SARCOMA DEVELOPMENT IN GRAFTED MICE.

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IN a previous communication (Barker, Dhar and Parsons, 1949) the effects on sarcoma growth in mice produced by treatment with some derivatives of purine and pyrimidine compounds were recorded. Further experiments described below have extended the range of pyrimidine compounds studied, and an attempt has been made to correlate molecular structure with biological activity. The compounds employed fall into the following groups :

(1) Cytidylic acid and allied compounds.
(2) Barbituric acid, its imino derivatives and related compounds.
(3) Acyclic analogues of some of the above compounds.

Pure line or stock mice were grafted with the homologous C57 or the heterologous S37 or Crocker sarcomas. The technique for treatment was similar to that previously described (Barakan, Barker, Gulland and Parsons, 1948), the standard dose (0·025 g.) being reduced in proportion to the toxicity of the particular compound. Heavy mortality occurred amongst mice treated with 2:4:6-triaminopyrimidine, 4:6-diaminopyrimidine and alloxan. Table I summarizes the results obtained. Statistical analysis has shown that the differences in tumour weights for treated and untreated grafted mice are significant for Groups 2, 10, 11 and 12. In all groups the number of controls equalled the number of treated mice.

(1) *Cytidylic acid and allied compounds.*

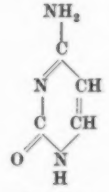
The effects on sarcoma growth in grafted mice treated with the four nucleotides of ribonucleic acid have been described in earlier reports (Parsons, Gulland and Barker, 1947 ; Barakan, Barker, Gulland and Parsons, 1948 ; Barker, Dhar and Parsons, 1949). In the course of these experiments it had been noted that the ratios of tumour weights in treated and control mice were not appreciably altered by substitution of the corresponding nucleoside or the free pyrimidine or purine for uridylic, adenylic or guanylic acids, the small differences in behaviour within each group being almost certainly due to differences in solubility in water. No such comparison was made, however, in the cytidylic acid series, since this nucleotide had been found to have little if any action on sarcoma development.

Recent observations (Brown, 1950 ; Hammarsten, Reichard and Saluste, 1950 ; Bendich, Getler and Brown, 1949) have shown that in rats fed with the pyrimidine nucleosides, cytidine or uridine, whereas cytidine is an effective pre-

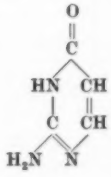
TABLE I.—*Total Tumour Weights of Treated and Untreated Mice.*

Compound.	Number of mice treated.	Total wt. of treated tumours. g. (T).	Total wt. of control tumours. g. (C).	Ratio T/C
1. Cystosine	12	39·0	38·1	1·00
2. Isocytosine	25	52·9	40·1	1·32
3. Arginine	19	40·8	41·0	0·99
4. Alloxan	38	73·4	76·2	0·96
5. Uramil	28	71·4	72·7	0·98
6. 5-Aminouracil (Group 1)	27	54·5	63·8	0·86
,, 5-Aminouracil (Group 2)	15	30·9	35·6	0·87
7. 2-Thio-4-amino-6-oxypyrimidine	10	19·7	22·4	0·87
8. Barbituric acid	16	27·1	28·6	0·95
9. 2-Amino-4:6-dioxypyrimidine	30	67·7	70·3	0·96
10. 4:6-Diamino-2-oxypyrimidine	25	28·6	56·0	0·51
11. 2:4-Diamino-6-oxypyrimidine	21	61·6	37·0	1·66
12. 2:4:6-Triaminopyrimidine	56	51·4	88·0	0·58
13. 4:6-Diaminopyrimidine	24	48·3	58·2	0·82
14. Acetamidine hydrochloride	27	48·6	46·3	1·04
15. Acetyl urea	35	46·0	51·9	0·88
16. Carbamido-acetamidine	19	33·7	28·4	1·18

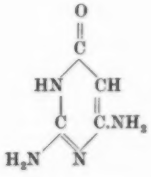
cursor of both the cytosine and uracil of nucleic acid, uridine is a relatively ineffective precursor of either. Cytosine and uracil, on the other hand, are not utilized in nucleic acid synthesis. This suggested that examination should be made of the action of cytosine when injected into grafted mice. A small number of sarcoma-bearing mice were therefore treated with this compound, which was found to have a negligible effect on tumour development. Isocytosine (2-amino-6-oxypyrimidine), on the other hand, in which the positions of the amino and oxy groups in cytosine are reversed, had a moderate acceleratory action. This finding is of interest, since the closely related compound 2:4-diamino-6-oxypyrimidine induces a similar, though more considerable, effect in grafted tumours, and this action becomes inhibitory again on reversing the amino and oxy groups in positions 2 and 6 of the molecule.



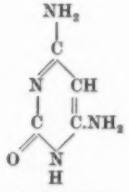
Cytosine.



Isocytosine.



2:4-Diamino-6-oxypyrimidine.



4:6-Diamino-2-oxypyrimidine.

It would seem from the above results that the cytosine molecule can exert no action when injected subcutaneously into grafted mice, whether it is administered as the free pyrimidine or combined with a ribose residue as in cytidylic

acid : the differences in metabolic behaviour between such compounds administered orally evidently have no bearing on their actions on tumours when injected.

There is evidence (Cerecedo, 1927) that cytosine administered orally to dogs is partly excreted unchanged and partly deaminated to uracil. Cytidine, on the other hand, is excreted as urea (Emerson and Cerecedo, 1930). Furthermore, after oral administration to rats, 87·3 per cent of cytosine nitrogen is excreted in six days (Bendich, Getler and Brown, 1949). In contrast to these examples of oral administration, subcutaneous or intraperitoneal injection of cytosine into various animals (Mendel and Myers, 1910) results in the excretion of the compound unchanged and no uracil was observed in the urine. In the present series of experiments no evidence has been obtained on the fate of the injected materials, but it is reasonable to assume that, in agreement with Mendel and Myers (1910), simple deamination of cytosine or cytidylic acid does not take place, since the products would have an acceleratory effect on tumour growth.

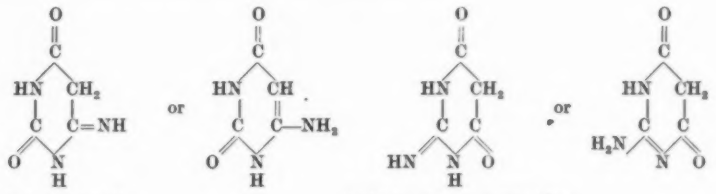
The fate of injected nucleotides is being examined by different methods and will be reported separately, but the problem has also been approached by a study of the effects on tumour growth of possible precursors and degradation products of purine and pyrimidine compounds. In a previous communication (Barker, Dhar and Parsons, 1949) 4-carboxyuracil (orotic acid), which has been reported (Hammarsten, Reichard and Saluste, 1949) as a precursor of uridine and cytidine, was found to be inactive when tested in grafted mice. Similarly arginine, which has been discussed (Hopkins, 1916) as a source of purine nitrogen, shows neither acceleratory nor inhibitory properties. Alloxan and uramil, which might also arise by oxidative degradation of purines and pyrimidines, have no effect on tumour development when injected. Injection of alloxan induced a characteristic red colour in the urine of the treated mice, suggesting that a proportion at least of the compound was excreted unchanged.

(2) *Barbituric acid, its derivatives and related compounds.*

As a possible interpretation of the earlier observation (Barker, Dhar and Parsons, 1949) that 4-aminouracil exhibits a significant inhibition of tumour growth, it had been suggested that this compound might act as a precursor of inhibitory bases, and in agreement with this view 4-methyluracil and 5-methyl-4-aminouracil, in which conversion to purine is blocked, were found not to have inhibitory properties. If the amino group is substituted in position 5 the resulting compound is also found to be without inhibitory activity. The behaviour of 4-aminouracil appears to be specifically associated with its chemical structure, since the introduction of the sulphur atom at position 2 to give 2-thio-4-amino-6-oxypyrimidine is sufficient to abolish its inhibitory action. It is considered possible that the marked difference in activity between 4-aminouracil and 5-aminouracil is due to the difference in the electron-availability at the free positions of the two molecules (positions 4 and 5 respectively), which would influence the ability of the tissues to metabolize the compounds by oxidation.

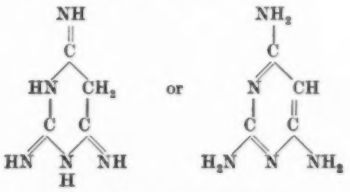
Since 4-aminouracil may also be regarded in its tautomeric form as 4-imino-barbituric acid, a systematic study was made of barbituric acid and its derivatives, all of which are characterized by high chemical reactivity at position 5. From the results shown in Table I it is clear that the barbituric acid structure cannot be responsible for the inhibitory action of 4-aminouracil. Barbituric acid injected

in tumour-bearing mice was found to be inactive, as was also 2-amino-4:6-dioxy-pyrimidine, which is isomeric with 4-aminouracil.



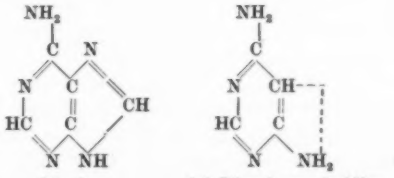
4-Aminouracil. *2-Amino-4:6-oxypyrimidine.*

Furthermore, the two isomeric di-imino-derivatives of barbituric acid differ markedly in their behaviour (see above), 4:6-diamino-2-oxy-pyrimidine having similar activity to 4-aminouracil, whereas 2:4-diamino-6-oxypyrimidine has a very considerable acceleratory effect. Bearing in mind the respective behaviour of the two mono-imino and the two di-imino derivatives of barbituric acid, it would seem that the 4-amino group, and to a lesser degree the 6-amino group, make a contribution to the biological action of these compounds diametrically opposed to that made by the imino group in position 2. It is not surprising, therefore, that 2:4:6-triaminopyrimidine has been found to have inhibitory properties, since in this molecule the effect of the 2-amino group is counterbalanced by the two " opposing " amino groups at positions 4 and 6.



2:4:6-Triaminopyrimidine.

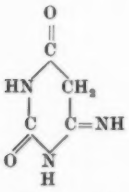
The inhibition of tumour growth observed with 4-aminouracil and with 4:6-diamino-2-oxypyrimidine does not appear, however, to be entirely due to the activity of the amino groups, since 4:6-diamino-pyrimidine exerts little if any inhibitory action, indicating that the 2-oxy group makes a positive contribution to the actions of the inhibitory compounds. The possibility has been put forward that 4-aminouracil can act as a purine precursor, and the presence of amino groups in positions 4 and 6 in the di-amino pyrimidine compound suggests from its similar structure that this compound may also play a part in adenine synthesis.



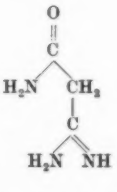
Adenine. *4:6-Diaminopyrimidine.*

(3) *The specificity of the heterocyclic ring.*

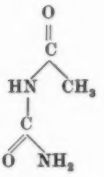
In the above discussion of the biological activity of the various constituent groups in the pyrimidine derivatives the structures were considered only as part of the heterocyclic system. In order to determine whether the ring structure of the compounds is essential to the activities observed three acyclic compounds have been tested. In the first, carbamido-acetamidine, the whole of the structure of 4-aminouracil is present except for the carbon atom at position 2. In the second, acetyl urea, the configurations at positions 1, 2, 3 and 6 of 4-aminouracil are imitated. The third, acetamidine, possesses a similar grouping of atoms to that at position 4 of 4-aminouracil. None of these compounds, when tested in sarcoma-bearing mice, were found to exert inhibitory activity, like 4-aminouracil or 4:6-diamino-2-oxypyrimidine.



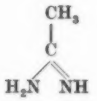
4-Aminouracil.



Carbamido-acetamidine.



Acetyl urea.



Acetamidine.

The production of shock.

In the use of compounds examined for their effects on tumour growth, difficulty in assessment of the value of the results is occasioned both by the toxicity of some of the compounds and also by the inducement of shock during treatment. It has already been stated that doses vary according to the condition of the treated mouse, and this must preclude any quantitative comparisons. There is also the possibility that apparent inhibitions of tumour development may be only a reflection of the general effects of a drug on the body tissues. It is significant, however, that when alloxan or barbituric acid is administered, although marked wasting and loss of total body weight occur, the tumours continue to grow with the same rapidity as those in the control mice.

It has been previously suggested (Barker, Dhar and Parsons, 1949) that the property of inducing shock shown by adenylic acid, adenosine and adenine is associated with the amino group at position 6. This generalization appeared to be confirmed when it was found that 4:6-diaminopyrimidine and 2:4:6-triaminopyrimidine, which possess part of the adenine structure, also produce shock on injection, though of a milder type than that caused by injection of adenine. It is noteworthy that no shock is induced on injection of 4-aminouracil or 4:6-diaamino-2-oxypyrimidine, which suggests that the presence of the 2-oxy group prevents the occurrence of shock. When it is considered, moreover, that hypoxanthine induces no shock as compared with that caused by adenine, it appears that substitution of an oxy group at position 6 is also effective in preventing shock. It seems, therefore, that the state of oxidation and/or amination of the pyrimidine ring may play an essential part in the prevention of shock.

SUMMARY.

(1) The effects on tumour development of a series of pyrimidine compounds injected into grafted mice have been examined. Of the compounds tested 4:6-diamino-2-oxypyrimidine and 2:4:6-triaminopyrimidine exhibit inhibitory activity. Reversal of the amino and oxy groups at positions 6 and 2 in the first-named compound reverses also the action of the drug and converts an inhibitory effect into an acceleratory. Similarly it has been found that reversal of the same groups in the cytosine molecule converts the negative action of this compound into a moderate acceleratory effect.

(2) The action on sarcoma growth of certain acyclic compounds injected in grafted mice and compared with those induced by 4-aminouracil have been found to be negative.

REFERENCES.

Barakan, T. H., Barker, G. R., Gulland, J. M., and Parsons, L. D.—(1948) *J. Path. Bact.*, **60**, 441.

Barker, G. R., Dhar, M. M., and Parsons, L. D.—(1949) *Brit. J. Cancer*, **3**, 427.

Bendich, A., Getler, H., and Brown, G. B.—(1949) *J. Biol. Chem.*, **177**, 565.

Brown, G. B.—(1950) *Fed. Proc.*, **9**, 517.

Cerecedo, L. R.—(1927) *J. Biol. Chem.*, **75**, 661.

Emerson, O. H., and Cerecedo, L. R.—(1930) *Ibid.*, **87**, 453.

Hammarsten, E., Reichard, P., and Saluste, E.—(1949) *Acta Chem. Scand.*, **3**, 432.
—(1950) *J. Biol. Chem.*, **183**, 105.

Hopkins, F. G.—(1916) *J. chem. Soc.*, 629.

Mendel, L. B., and Myers, V. C.—(1910) *Amer. J. Physiol.*, **26**, 77.

Parsons, L. D., Gulland, J. M., and Barker, G. R.—(1947) *Symp. Soc. exp. Biol.*, **1**, 179.

ASPECTS OF THE CHEMISTRY OF THE PROTEINS IN THE NUCLEUS; A SHORT REVIEW AND SOME EXPERIMENTAL RESULTS.

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FIFTY years ago various workers, notably Miescher (1897) and Kossel (1896), first extracted material which they termed nucleo-protein and Kossel (1928) carried out investigations on the protein moieties of this substance. In some germ cells (e.g. salmon milt) there appeared to be a simple protein in combination with the nucleic acid which could readily be isolated by virtue of its basic character. Kossel (1928) described similar protein material from various mammalian cells (e.g. calf thymus lymphocytes) which also had basic properties, though a more complicated chemical structure. The two classes of proteins have been termed the protamines and histones, respectively.

These proteins, in association with nucleic acid, i.e. nucleoprotein, are believed to play an important part in cell reproduction, but their basic functions have not been elucidated. Recently developed analytical techniques, particularly chromatographic analysis and the use of the ultra-violet spectrophotometer, have produced considerable fresh data on the composition of the nucleic acids, but much less research has been done on the protein fractions. This paper collects some information on this topic and some analytical results obtained in our laboratory are presented.

The Proteins Found in the Nucleus.

Mirsky and Pollister (1946) were able to dissolve nuclear nucleoprotein in 1 M sodium chloride, after first removing the cytoplasmic material with 0·14 M saline, and to isolate this nucleoprotein by diluting the solution to a salt concentration of 0·14 M, when it precipitates in fibrous form. Similar material could be obtained from isolated nuclei (Dounce, 1943) confirming that these nucleoproteins were of nuclear origin. This extracted material was found to consist of deoxyribonucleic acid and two protein fractions, one of which was classified as a histone, being readily soluble in acid and precipitated out of solution by ammonia. The second protein was characterized by having a higher tryptophane content and being soluble in acid or alkali, and was referred to as the tryptophane-containing protein (Tr.pr.).

Later these observations were extended and it was found that by drastic mechanical treatment of the nuclei followed by differential centrifugation, a material could be obtained consisting of small fibres having certain characteristics similar to chromosomes, when examined under the microscope (Mirsky and Ris, 1947; Mirsky, 1947). The conclusions have, however, been questioned by

Lamb (1949), who maintained that under the conditions employed the nuclei are actually torn into fibres, but not necessarily ruptured. Mirsky (1947) found that treatment of the isolated chromosomes with 1 M sodium chloride caused much of the material to pass into solution, as had been observed with the isolated nuclei. Thread-like portions, termed "residual chromosomes," remained undissolved, and contained protein with some nucleic acid. The material extracted was a nucleoprotein containing nucleic acid and histone, but no Tr.pr. It was suggested, therefore, that this must be derived from the residual chromosomes. It must be pointed out, however, that the method which successfully extracted nucleoprotein containing Tr.pr. from isolated nuclei, apparently failed to do so from the chromosomes, and therefore claims respecting the identity of Tr.pr. and the residual chromosomes must await analytical confirmation. It is of special interest that cells which could be considered metabolically active (liver, kidney) contain as much as 50 per cent of the total isolated chromosome material in the form of residual chromosome.

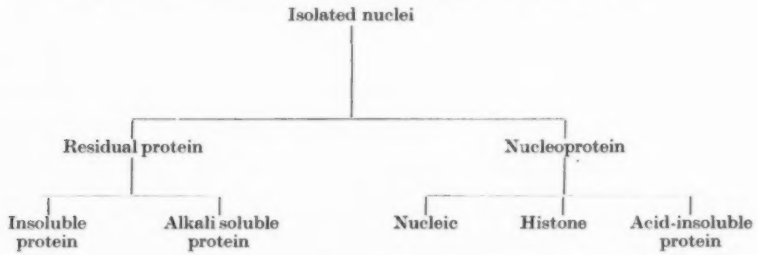
Stedman and Stedman (1943, 1947) also isolated an acid-insoluble protein from the nucleus which they termed "chromosomin," believing that it was the structural protein of the chromosome and that in the nucleus it was embedded in a support of nucleo-histone. The work of both Mirsky (1947) and Stedman and Stedman (1943, 1947) seems to indicate that a protein forms the basic structure of the chromosomes though the part played by the nucleic acid and the histone is uncertain. This is an important aspect but cannot be discussed here. Mention should be made, however, of the work of Mazia (1941) and Mazia, Hyashi and Yudowitch (1947), who found that enzymatically the nucleic acid can be digested away from salivary gland chromosomes without disrupting the structure, but that a proteolytic enzyme would cause disintegration. Kauffmann, Gay and MacDonald (1949) have recently suggested that this work may need further investigation before interpretations on chromosome structure can be made.

Mayer and Gulick (1942) prepared nuclear material by a method in which the tissue is frozen, dried and then fractionated by sedimentation in organic solvents at various specific gravities. From this material they isolated nucleic acid and histone, and in addition a fraction with isoelectric point between 5·8 and 6·15, which constituted an appreciable proportion of the nuclear proteins. The evidence suggested that the fraction contained a sulphur-containing protein with an acidic isoelectric point and a protein of globulin type. If this material was, indeed, of nuclear origin, then this fraction appears to correspond in properties with the "chromosomin" of Stedman and Stedman (1943, 1947). Recently Wang, Kirkham, Dallan, Mayer and Thomas (1950) have reported the isolation of a similar acidic-protein fraction from rat liver nuclei and from calf thymus chromosomes. The nuclei and chromosomes were first extracted with 1 M sodium chloride and then the residue was extracted with dilute sodium hydroxide. Acidification of this extract precipitated a protein which contained about 6 per cent arginine and no nucleic acid. Since the nucleoprotein extracted from the nuclei has been shown to contain nucleic acid histone and Tr.pr., the alkali soluble protein here must be of different character and this is more likely to be the protein of the residual chromosomes than the Tr.pr. fraction. Finally, two Russian workers (Zbarskii and Debov, 1948) have reported that the protein remaining after the extraction of the nucleoprotein consists of two fractions, one of which is weakly acidic in character, being soluble in dilute alkali and having an isoelectric point

at pH 5·0 to 5·3 (presumably similar to the fraction reported by Wang, Kirkham, Dallan, Mayer and Thomas (1950)), and secondly a final residue which is insoluble in all the usual extraction media and only swells in alkali.

In the nucleus, therefore, there is evidence of the presence of four protein fractions, in addition to the nucleic acid. Two components are extracted by 1 M sodium chloride in association with the nucleic acid, namely, the histone and an acid-insoluble protein. The protein of the nucleus remaining undissolved after the extraction can then be split into two parts, a fraction soluble in dilute alkali and a remaining insoluble residue (Fig. 1). Further work will be necessary before the role and location of these fractions in the nucleus can be ascertained.

FIG. 1.—The different fractions reported to be present in the nucleus.



It has now been shown that the nucleus has its own specific pattern of enzymes and since these are, presumably, protein in character, they must be considered. Certain enzymes of the liver (e.g. arginase, D-amino-oxidase) have been found to be present in greater concentration in the nucleus than in the whole liver; alkaline phosphatase is also very abundant, while others, particularly cytochrome oxidase and succinic dehydrogenase, have been found to be much lower. Indeed, with liver cell nuclei, the absence of succinic dehydrogenase activity has been used as a criterion of purity of the preparations. If confirmed, this may possibly have an application to nuclear protein work in proving that only nuclei are being used and so avoiding any cytoplasmic contamination. These aspects have been well reviewed by Dounce (1950).

Available Analytical Data.

Most of the analytical results reported in the literature concern the protein histone; so far no complete quantitative amino-acid analysis of this or any of the other fractions is available, with the exception of the data of Kossel (1928) summarized in Table I. Arginine was found to be the main constituent, accounting for some 25 per cent of the protein nitrogen, with leucine and lysine next in content.

Felix and Harteneck (1926, 1927) and Felix and Rauch (1931) in a series of papers described the preparation and some analytical investigations on thymus histone. Analyses were reported on histone hydrochloride and a possible minimum molecular weight of 14,200 was estimated. The hydrochloride contained 15·9 per cent nitrogen and 1·87 per cent sulphur, 25·7 per cent of the nitrogen being due to arginine and 5·5 per cent to histidine. In other work it was found that the histone had 20 basic groupings and 7·5 free-COOH groups per 100 atoms

TABLE I.—*Early Results of the Amino-acid Composition of Thymus Histone (Kossel, 1928).*

The values are minimal and are expressed as a percentage of the total nitrogen.

Alanine	3·46	Leucine	11·8
Ammonia	6·46	Lysine	8·04
Arginine	25·17	Phenylalanine	2·20
Glutamic acid	0·53	Proline	1·46
Glycine	0·50	Tyrosine	5·20
Histidine	1·79		

nitrogen, while the changes produced by pepsin treatment indicated the presence of carboxyl-guanidine (or arginine) linkages.

Euler and Hahn (1946) investigated nucleo-protein from isolated nuclei and found that, with their preparative technique, some 45 per cent of the nucleo-protein passed through the membrane on dialysis against 1 M sodium chloride. Since this did not occur with nuclei dialysed in water it was presumed that the salt solution had produced some dissociation. However, when the nucleoprotein was prepared directly from thymus gland, only a very small proportion was dialysable, so presumably some degradation must have occurred during the isolation of the nuclei. In a later paper in this series Ahlstrom (1947) reported that 40 per cent of histone hydrochloride had a molecular weight less than 10,000. This phenomenon has not been reported by any other workers and in the absence of any analytical characterization of the materials is difficult to assess. Davidson and Lawrie (1948), using paper chromatography, carried out qualitative analyses of specimens of histone and non-histone (acid-insoluble) proteins from nuclei of calf thymus, rat liver and fowl erythrocytes. The histones contained alanine, arginine, aspartic acid, glutamic acid, isoleucine, leucine, lysine, phenylalanine, proline, serine, tyrosine, valine and one unidentified constituent. In addition to the above amino-acids the acid-insoluble portein contained 1 per cent tryptophane, glycine but no lysine, and a further unidentified component. In similar studies Khouvine and Gregoire (1949) have analysed nuclear nucleoprotein from rat epithelioma and compared it with that found in the surrounding nectrotic tissues. The acid-extractable proteins were compared, using paper chromatography and found to be qualitatively the same. Very recently Stedman and Stedman (1950) have reported arginine contents of histones from various tissues and found they all contained about 28 to 30 per cent arginine (as per cent of total nitrogen). In addition these workers claim to have isolated sub-fractions of the histone of different composition, but no details of the method of extraction or fractionation are given.

Importance has often been attached to the tryptophane content of the nuclear proteins. Generally speaking, histone specimens have been found to contain 0·0 per cent to 0·1 per cent, while the acid-insoluble proteins have about 1·0 per cent tryptophane. Stedman and Stedman (1947) believe that the presence of tryptophane in histone is an indication of impurity. Mirsky and Pollister (1946) found very small amounts of tryptophane in histone, but about 0·8 per cent in the Tr.pr. fraction, and 1·36 per cent in the residual chromosomes. Felix and Rauch (1931) and Stedman and Stedman (1947) have both found sulphur and SH-groupings in histones, though other workers have not reported this.

Biological Properties.

There is little precise information on the biological function of the proteins in the nucleus except that they are believed to be vital to cell reproduction. Using micro-spectrophotometry, Caspersson (1950) has studied the content and distribution of the nucleoproteins in the cell both at interphase and during mitosis. This method has given very interesting data on the distribution of the nucleic acids, but less certain data on the distribution of the proteins, since the absorption spectra of different proteins do not vary considerably with their composition. From measurements of light absorption at 2800 Å, Caspersson claims to have found changes in the amount of histone present at different stages in mitosis. The investigations in this field have recently been summarized by Caspersson (1950). Of the other histochemical studies reference will only be made to the recent work of Thomas and Steinitz (1950) using a method which will stain proteins of high arginine content in paraffin sections. The application of this method has indicated that the ratio of arginine-containing protein (histone type) to total protein, is greater in normal epidermis. Possibly a combination of this technique with spectrophotometric methods will give further data on the localization of the basic proteins in the nucleus.

Extracted histone has been tested for bactericidal action (Weissman and Graf, 1947) and the pharmacological properties of histone from avian erythrocytes have also been studied. The toxicity to mice and rabbits has been reported as 255 mg./kg. (LD. 50) for histone sulphate (Reiner, De Beer and Green, 1942). Some inhibitory action on grafted mouse Carcinoma 2146 has been described (Stedman, Stedman and Pettigrew, 1944).

THE PRESENT INVESTIGATIONS.

These have been reported in part to the Vth International Cancer Congress, Paris (Hamer, 1950).

In this report the analysis of histone from two sources will be described along with some other observations on this material and its isolation. The work is continued and extended to establish the characteristics of the different protein fractions obtained from various tissues. Related work from these laboratories on the nucleic acids in the nucleus and cytoplasm has already been reported (Woodhouse, 1949, 1950).

Preparation of Material.

The two tissues used for this work were: (*a*) Calf thymus gland, (*b*) transplantable rat sarcoma, originally induced by a methylcholanthrene pellet implanted in the leg muscle. Nucleoprotein was extracted from these tissues in the manner described by Mirsky and Pollister (1946). The nucleoprotein extract was dissolved and precipitated three times by alternate solution in 1 M sodium chloride followed by dilution to 0·14 M. The final precipitate was extracted overnight with 0·1 N hydrochloric acid in the cold. The extract was dialysed free from acid and then the histone precipitated with ammonia. The precipitate was centrifuged off and dissolved in acid, centrifuged and dialysed against distilled water and lyophilised. The resulting protein was a white, light powder

which dissolved readily in the presence of a trace of acid. The nitrogen content of histone from both the above materials was 18·2 per cent.

The acid-insoluble protein was prepared from the whole protein of the nucleoprotein, separated from the nucleic acid by the method of Sevag, Lackman and Smollens (1938). The whole protein was washed free of nucleic acid by shaking with more 1 M sodium chloride and then extracted with 0·1 N hydrochloric acid. The histone dissolved and the remaining insoluble protein was dialysed and dried. For dialysis the protein fractions were hydrolysed for 18 hours under reflux, with 6 N hydrochloric acid (redistilled).

Qualitative analysis.

After hydrolysis the protein fractions were examined by paper chromatographic analysis. The solvent n-butanol-acetic acid-water (4:1:5) was used, followed by either collidine-lutidine-water (1:1:4) or phenol-water. The papers were sprayed with 0·2 per cent ninhydrin and heated for 6 minutes at 100° C. to develop the characteristic ninhydrin-amino-acid colours. Reference mixtures were always run at the same time.

In the acid-hydrolysates of three specimens, histone and the acid-insoluble protein from thymus gland and histone from rat sarcoma, exactly the same components were found. Indeed, by this qualitative method it was not possible to detect any differences in composition in three fractions. All contained the following amino-acids: Alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine and leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, valine. No sulphur-containing amino-acids were detected. The presence of histidine was confirmed with the Pauly reagent. The analyses differ from the results presented by Davidson and Lawrie (1948) in that those workers did not find any glycine, histidine or threonine in histones. The chromatographic analyses on starch columns described below did not reveal any further components but confirmed the presence of those listed.

Quantitative analysis.

The individual amino-acids in the protein hydrolysates were first separated by chromatography on starch columns and then estimated colorimetrically with ninhydrin as described by Moore and Stein (1948, 1949). The hydrolysates were evaporated to very small volume under reduced pressure and then taken up in a small volume of the solvent (*a*) below. Small samples (0·5 c.c.) containing approximately 0·45 mg. nitrogen were used for each analysis except in the case of the solvent (*c*), when half this amount was used to avoid excess of water on the column. Columns of potato starch 1 cm. diameter and 30 cm. long were used in conjunction with the following solvents: (*a*) n-butanol-n-propanol-0·1 N HCl (1:2:1) followed by n-propanol-0·5 N HCl (2:1); (*b*) ter-butanol-sec-butanol-0·1 N HCl (2:1:1); (*c*) benzyl alcohol-n-butanol-water (1:1:0·25). These solvents resolved all the amino-acids found in the hydrolysates, so that quantitative estimations on the column effluent could be made. The effluent was collected in fractions of about 0·7 ml. every 35 minutes, when the column was run under positive pressure of 9 cm. mercury, using a time-based fraction collector (Hough, Jones and Wadman, 1949). The whole fraction was taken for analysis with 1 ml. of 2 per cent ninhydrin in methyl-cellosolve-citrate buffer (1:1, pH 5) containing 0·8 per cent

stannous chloride. The ninhydrin-ammonia colour was produced by heating in a boiling water bath for 20 minutes and then, after dilution to 8 ml. with a propanol-water (1:1) mixture, measured in the Hilger "Spekker" absorptiometer using Ilford spectrum yellow filter H 606 (proline with violet filter H 601). The colour values of the tubes covering the emergence of an amino-acid were added together and the total amount present was then calculated, making allowance for the variable colour yields of the amino-acids (Moore and Stein, 1949). The results for thneonine and serine were corrected for decomposition during hydrolysis according to the work of Rees (1946). The results were expressed as percentages of the hydrolysate nitrogen taken and good recoveries of this nitrogen and ammonia were obtained. Moore and Stein (1949) studied the recovery of individual amino-acids from mixtures and found that the chromatographic procedure on starch columns is capable of yielding recoveries of 100 $\pm$ 3 per cent. Their average recoveries were well within this range and the sum of the amino-acids was almost invariably accurate to $\pm$ 1 per cent. The results for the major components in Table II are estimated to be within $\pm$ 3 per cent accurate, while the values for certain minor components (histidine, proline and tyrosine) are probably accurate to $\pm$ 5 per cent with the experimental technique used.

The results for the two histone specimens from calf thymus and rat sarcoma are given in Table II.

TABLE II.—*Amino-acid Analysis of Histones.*

	A.	B.
Alanine	6·0	4·7
Ammonia	4·8	5·1
Arginine	30·7	29·1
Aspartic acid	3·3	3·9
Glutamic acid	2·25	1·75
Glycine	5·2	6·9
Histidine	4·0	3·4
Isoleucine	12·0	10·3
Leucine	3·05	3·0
Lysine	10·8	12·5
Phenylalanine	1·9	2·4
Proline	2·7	2·4
Serine	3·45	3·9
Threonine	3·1	4·4
Tyrosine	1·4	1·1
Valine	4·9	5·1
	99.55	99.95

Amino-acid nitrogen as percentage of protein nitrogen. A, From calf thymus; B, from rat sarcoma.

The results show that arginine is the major constituent accounting for some 30 per cent of the protein nitrogen (Stedman and Stedman, 1950), while the other basic amino-acids, lysine and histidine, account for about 11 per cent and 3·5 per cent respectively. Isoleucine is the other principle constituent and the ratio

of isoleucine to leucine (4:1) is the reverse of that found in many other proteins (e.g. insulin, albumin, γ-globulin). The basic protein, protamine, from germ cell nucleoprotein contains isoleucine but no leucine. By separate analysis no appreciable amount of trytophane could be found in the histone specimens. Less than 0·04 per cent was found using the colorimetric method described by Spies and Chambers (1948). No sulphur-containing amino-acids were detected chromatographically, though using the Brdicka (1934) polarographic test about 0·04 g. cyst(e)ine per 100 g. protein was indicated in the rat sarcoma histone. The thymus histone specimen was quite negative in this test. Comparing the two specimens analysed, there are no striking differences in composition. The amounts of threonine and glycine are some 30 per cent higher in the sarcoma specimen, while the alanine content is 20 per cent lower. The total amounts of the basic (arginine, lysine, histidine) and acidic (glutamic and aspartic acids) amino-acids are the same in both proteins.

Analysis of the acid-insoluble protein.

As explained earlier, no difference in composition could be detected between histone and the acid-insoluble protein of thymus nucleoprotein by qualitative chromatographic analysis. Preliminary quantitative analyses on one specimen have not revealed any appreciable difference in the proportions of the 15-amino-acids present as compared with those in thymus histone. However, when hydrolysates were tested by the polarographic method, an appreciable catalytic wave was obtained, indicating the presence of approximately 0·5 per cent cyst(e)ine in the protein. It would, therefore, appear that the difference in the properties of the two protein fractions, histone and the acid-insoluble protein, is due simply to the presence of cystine (or cysteine) in the latter, perhaps producing some different arrangement or linkage of the molecules to give an insoluble structure. One other minor point of difference observed was that, whereas acid hydrolysates of histone were a clear pale yellow, those of the acid-insoluble protein were much darker and contained some insoluble black " humin " material.

Other experimental observations.

Histone was very easily extracted from thymus tissue in appreciable amounts by the technique reported earlier. However, using the same method only a comparatively small amount was obtained from the rat sarcoma material while, up to the present, yields obtained from transplanted mouse tumours (a " C3H carcinoma " and a chemically induced transplantable sarcoma) have been too small to permit characterization. Instead, it has been found that hydrochloric acid extracted a protein which precipitated on dialysis against water, but redissolved in acid, apparently having an isoelectric point about pH 6. It is not yet clear whether this is a fraction of different character or simply a denatured histone product. Histone appears to denature very easily, thus although the material is very soluble in dilute acid after lyophilizing, in less acidic solutions (pH 5·5 to 7) it disperses much more slowly and gives an opalescent solution.

One specimen of histone was taken up in phosphate buffer of pH 6·5 when a slightly opalescent solution resulted. This solution was then tested on the analytical ultracentrifuge and showed the presence of a high molecular weight fraction sedimenting very rapidly, followed by a lighter material with sedimen-

tation constant $1{\cdot}99 \times 10^{-13}$ at 20° C., corresponding to a molecular weight of about 15,000. Since no nucleic acid could be detected the heavier material was presumed to be denatured histone and the other fraction possibly true histone.

DISCUSSION.

It is remarkable that no considerable differences have so far been found between the chemical composition of the histone and the acid-insoluble protein of nucleoprotein. The latter has, however, been found to contain a small amount of cystine or cysteine. Stedman and Stedman (1943) believed that their acid-insoluble material (chromosomin), despite a high content of the basic amino-acids must also contain a relatively large amount of glutamic acid. The present analyses do not support this claim. Since the two proteins, histone and the acid-insoluble type are so similar and are extracted together, it seems possible that they may be bonded together in the cell and are subsequently split by the extraction techniques to give basic soluble histone and a denatured " sulphur-histone " product.

No considerable differences have as yet been found in the nuclear proteins of normal and neoplastic tissues. There are some small differences in the amino-acid compositions and there is evidence of variation in the proportions of the protein fractions in different tissues. This may be connected with Mirsky's (1947) observation on the variation of the proportion of " residual chromosomes " in the cell in relation to its metabolic activity. More analytical data is required on the other fractions referred to in the review section and this aspect is being pursued.

SUMMARY.

1. Previous work on the proteins of the nucleus of mammalian tissues is reviewed.

2. Complete amino-acid analyses of histone from calf thymus and from rat sarcoma are given. Only small quantitative differences were found, but there is evidence of a difference in the proportion of the protein components in different materials.

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REFERENCES.

Ahlstrom, L.—(1947) *Ark. Kemi Min. Geol.*, **A24**, No. 31.
Brdicka, R.—(1934) *Biochem. Z.*, **272**, 104.
Caspersson, T.—(1950) ' Cell Growth and Cell Function.' New York (Norton & Co.).
Davidson, J. N., and Lawrie, R. W.—(1948) *Biochem. J.*, **43**, 29.
Dounce, A. L.—(1943) *J. biol. Chem.*, **147**, 685.—(1950) *Ann. N. Y. Acad. Sci.*, **50**, 982.
Euler, H. v., and Hahn, L.—(1946) *Ark. Kemi Min. Geol.*, **A22**, No. 17 ; **A23**, No. 5.
Felix, K., and Harteneck, A.—(1926) *Z. physiol. Chem.*, **157**, 76.—(1927) *Ibid.*, **165**, 103.
Idem and Rauch, H.—(1931) *Ibid.*, **200**, 27.
Hamer, D.—(1950) *Abstracts Vth int. Cancer Congr., Paris*, p. 100.

HOUGH, L., JONES, J. K. N., AND WADMAN, W. H.—(1949) *J. chem. Soc.*, 2511.
KAUFFMAN, B. P., GAY, H., AND MACDONALD, M. R.—(1949) *Cold Spring Harbor Symposia on Quantitative Biology*, **14**, 85.
KHOUVINE, Y., AND GREGOIRE, J.—(1949) *C. R. Soc. Biol., Paris*, **228**, 1167.
KOSSEL, A.—(1896) *Z. physiol. Chem.* **22**, 176.—(1928) 'The Protamines and Histones.' London (Longmans).
LAMB, W. G. P.—(1949) *Nature*, **164**, 109.
MAYER, D. T., AND GULICK, A.—(1942) *J. biol. Chem.*, **146**, 433.
MAZIA, D.—(1941) *Cold Spring Harbor Symposia on Quantitative Biology*, **9**, 293.
Idem, HAYASHI, T., AND YUDOWITCH, K.—(1947) *Ibid.*, **12**, 112.
MIESCHER, F.—(1897) *Die histochemischen und physiologischen Avbeiten* **1**, 2 : Leipzig (Vogel).
MIRSKY, A. E.—(1947) *Cold Spring Harbor Symposia on Quantitative Biology*, **12**, 143.
Idem AND POLLISTER, A. W.—(1946) *J. gen. Physiol.*, **30**, 117.
Idem AND RIS, H.—(1947) *Ibid.*, **31**, 1.
MOORE, S., AND STEIN, W. H.—(1948) *J. biol. Chem.*, **176**, 367.—(1949) *Ibid.*, **178**, 53.
REES, M. W.—(1946) *Biochem. J.*, **40**, 632.
REINER, L., DE BEER, E. J., AND GREEN, M.—(1942) *Proc. Soc. exp. Biol., N. Y.*, **50**, 70.
SEVAG, M. G., LACKMAN, D. B., AND SMOLLENS, J.—(1938) *J. biol. Chem.*, **124**, 425.
SPIES, J. R., AND CHAMBERS, D. C.—(1948) *An. Chem.*, **20**, 30.
STEDMAN, E., AND STEDMAN, E.—(1943) *Nature*, **152**, 267.—(1947) *Cold Spring Harbor Symposia on Quantitative Biology*, **12**, 224.—(1950) *Nature*, **166**, 780.
Iidem AND PETTIGREW, F. W.—(1944) *Biochem. J.*, **38**, xxxi.
THOMAS, L. E., AND STEINITZ, L. M.—(1950) Cancer Res., **10**, 245.
WANG, T., KIRKHAM, W. R., DALLAN, R. D., MAYER, D. T., AND THOMAS, L. E.—(1950) *Nature*, **165**, 974.
WEISSMAN, N., AND GRAF, L. H.—(1947) *J. infect. Dis.*, **80**, 145.
WOODHOUSE, D. L.—(1949) *Brit. J. Cancer*, **3**, 510.—(1950) *Abstracts Vth int. Cancer Cong., Paris*, p. 100.
ZBARSKII, I. B., AND DEBOV, S. S.—(1948) *Dokl. Akad. Nauk. S.S.S.R.*, **63**, 795 (*Chem. Abstr.* **43**, 2652 (1949)).

STUDY OF THE IODOACETATE INHIBITION OF THERMOCOAGULATION OF SERUM PROTEIN IN CANCER AND TUBERCULOSIS.

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HUGGINS, Miller and Jensen (1949) studied the thermocoagulability of the serum protein in health and disease in the presence of an extrinsic inhibitor—iodoacetic acid. Their results, however, while showing an abnormal behaviour for malignant and certain non-malignant diseases, tend to establish a seeming relationship between cancer and tuberculosis. The purpose of this paper is to clarify the difference in behaviour of the serum from cancer and tuberculosis patients in determining the type of " Index." The selection of tuberculosis serum was purely fortuitous. It arose from a desire for a " positive " control, as well as a negative, because the tuberculosis serum was supposed to resemble cancer in this test. From the experiments described here it would appear that a " positive " test encountered in tuberculosis does not apparently reflect the identical conditions of the blood serum responsible for a " positive " test in the case of a number of early, new and untreated cancer patients. This report is not an evaluation of the degree of accuracy of the method as a cancer diagnostic test.

METHOD AND MATERIAL.

The test was carried out exactly as described by Huggins, Miller and Jensen (1949), except for a modification as suggested by them (private communication). This was the use of a wire loop, 11 to 12 mm. in diameter, that was quickly thrust into each test-tube after boiling the sera, to loosen the " tenacious " layer of film on top of the coagulum. The tube was inclined to approximately a 45° angle, to evaluate the degree of clotting or liquification. Difficulties in " reading " the degree of clotting were mainly encountered in the new cancer cases. That is, there appeared to be a higher incidence of transitional zones of the " shapeless viscous mass " type, than the clear-cut changes from clot to complete liquid that prevailed for the tuberculosis sera, the advanced treated cancer cases, and the majority of benign tumour subjects and normal healthy persons. Readings were done by two persons with agreement on identical tubes as to clots or complete liquification, and agreement within either of two tubes when considering the " shapeless viscous mass " type of coagulum. The factor which differentiates one tube from another is that of the micromoles of sodium iodoacetate. This, divided by the reciprocal serum protein per cent, serves to fix the type of " index," or :

$$\text{Iodoacetate Index (I.I.)} = \frac{0{\cdot}5 \times 2 \times \text{m}}{\%\ \text{protein}}$$

where 0·5 = ml. serum, m = micromoles of sodium iodoacetate in the nearest tube containing a firm clot preceding a tube in which the coagulum is a " shapeless viscous mass," or in the original state before boiling.

The serum protein determinations were carried out by the semi-micro Kjeldahl procedure recommended by Huggins, Miller and Jensen (1949). Every fifth determination was duplicated with an average agreement of ± 0·5 per cent. The procedure was frequently checked for accuracy for the recovery of 5·0 mg. quantities of nitrogen by carrying out a complete determination with 23·6 mg. quantities of anhydrous ammonium sulfate with recovery in duplicate being ± 0·02 to 0·03 mg. of nitrogen.

The source of the tuberculosis blood samples was the Harbor Hospital, Torrence, and the cancerous, benign neoplasia, and non-symptomatic blood samples were from our own clinic. All the tests reported here were done in the order in which they were received. For purposes of analysis the cases are regrouped as follows :

Group I consists of 46 biopsied, new, untreated cancer cases.

Group II consists of 36 advanced cancer cases in various stages of the disease.

Group III consists of 55 pulmonary tuberculosis cases, of which 11 were arrested.

Group IV consists of 92 biopsied benign growth-bearing patients.

Group V consists of 85 non-symptomatic individuals.

The thermocoagulation : per cent serum protein relationship of the various groups is represented graphically in Fig. 1.

It is necessary to comment on the clinical status of the various groups of subjects. All tests were performed after the diagnosis had been established. All the Group I patients were still in relatively good physical condition. None of these cases tested were followed up for repeat determinations, except the two comedocarcinomas, which were done before surgery. None of the treated cancer cases were H.M.J. tested (as we shall now refer to the test, since it was so designated by the authors) before treatment. The two myelomas were receiving nitrogen mustards ; the leucaemias, X-rays and/or transfusions ; some of the advanced cases, polysaccharides or chymotrypsin ; eight of the breast cancer cases, testosterone. None of the patients in Groups I and II received any special diet, except that the most advanced, uncontrolled cases were getting either plasma transfusions or protein hydrolysates of one kind or another. All of them received an iron-vitamin preparation. One of the 11 negative cases in Group III had an acid-fast sputum at the time the H.M.J. test was done.

EXPERIMENTAL RESULTS.

Consideration of Table I shows the correlation of the serum protein per cent with the I.I., as representative of the various groups of cases. Only 24 out of 46 or approximately 52 per cent of the Group I cases demonstrated a positive H.M.J. (index less than 9·0), whereas 23 out of 36, or 63 per cent, of Group II, and 44, or 80 per cent, of Group III, also yielded a positive H.M.J. At first hand it would appear that the test was far more " efficient " in diagnosing tuberculosis than cancer. However, it will be noted that the I.I. was derived from protein

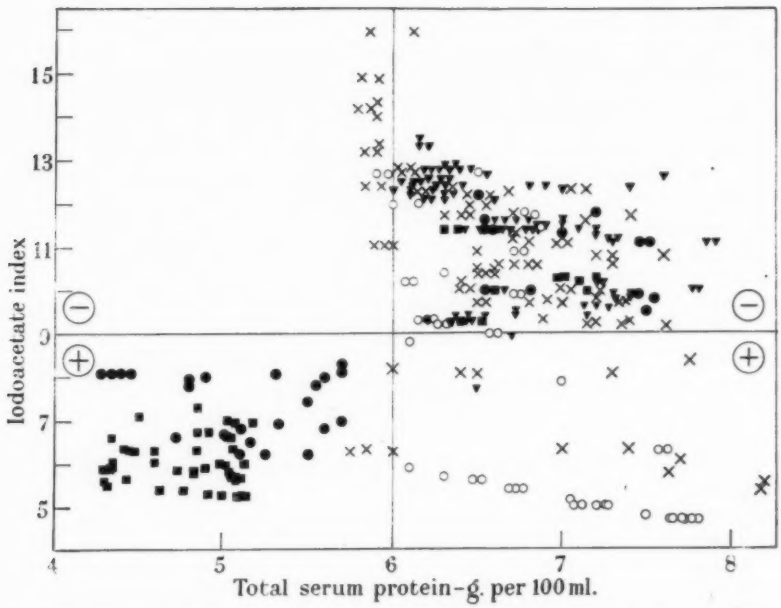

FIG. 1.—Distribution of iodoacetate indices and protein content of serum. The horizontal line represents the protein values in g. per 100 ml. serum, and the vertical line the corresponding indices, drawn from data. ⊕ = positive segment; ⊖ = negative segment of correlation. ○I, untreated cancer cases; ●II, advanced cancer cases; ■III, pulmonary tuberculosis; ×IV, benign neoplasms; ▼V, non-symptomatic controls.

TABLE I.—*Summary of the Correspondence of Iodoacetate Index Range with the Total Protein Range.*

Subjects.	Number of cases.	Iodoacetate index.	Total protein per cent.
Group I: Early cancer	24	4·7– 8·8	6·1–7·7
,, ,,	22	9·0–13·8	5·9–6·8
,, II: Advanced cancer, treated, uncontrolled	23	6·2– 8·1	4·4–5·7
Advanced cancer, treated, controlled	13	9·5–12·2	6·6–7·5
,, III: Active pulmonary tuberculosis	44	5·3– 7·3	4·3–5·1
Arrested pulmonary tuberculosis	11	9·3–11·4	6·3–7·2
,, IV: Benign neoplasia	78	9·3–16·0	5·9–7·6
,, ,,	14	5·4– 8·4	5·8–8·1
,, V: Non-symptomatic normals	83	9·3–11·3	6·2–8·2
,, ,,	2	7·7– 8·9	6·5–6·7

values well within the normal range for this procedure in Group I, while virtually the same I.I. range for Groups II and III was based upon a hypoproteinemia. However, in those instances where the result was a negative H.M.J., regardless of the clinical state, there was no such discrepancy in the serum protein values.

The observed variations in the properties of the serum are detailed in Table II, and Table III is a breakdown of the types of growth encountered with indices greater or less than 9·0.

A minor but interesting point emerging from our work is the fact that the numerical values of the positive range of indices for Groups I and II is not due

TABLE II.—*Frequency of Occurrence of Various Iodoacetate Indices in Different Conditions.*

Iodoacetate. index.	Group I. Untreated cancer. 46 cases. (Per cent.)	Group II. Advanced cancer. 36 cases. (Per cent.)	Group III. Pulmonary tuberculosis. 55 cases. (Per cent.)	Group IV. Benign neoplasia. 92 cases. (Per cent.)	Group V. Non-symptomatic controls. 85 cases. (Per cent.)
4·6– 5·0	26·1	—	—	—	—
5·1– 6·0	17·4	—	47·3	3·2	—
6·1– 7·0*	4·3	27·8	29·1	6·5	—
7·1– 8·0	2·2	16·7	3·6	—	1·1
8·1– 9·0	6·5	19·4	—	5·4	1·1
9·1–10·0	15·2	13·9	7·3	18·5	26·2
10·1–11·0	10·9	—	7·3	24·0	2·4
11·1–12·0	10·9	19·4	5·4	14·2	26·2
12·1–13·0	6·5	2·8	—	16·3	39·4
13·1–14·0	—	—	—	4·3	3·6
14·1–15·0	—	—	—	5·4	—
15·1–16·0	—	—	—	2·2	—

* This range of indices includes the average values of the two comedocarcinoma cases.

to the severity or extensiveness of the malignant process. Despite the so-called "precancerous" nature of the two comedocarcinomas in Group I, the degree of "positiveness" was greater than for the lymphosarcoma and primary lung adenocarcinoma (I.I. between 8 and 9). The differential nature of the I.I. for the series of cases in Group IV merely reflects the ratio of concentration of iodoacetate as an inhibitor of coagulation for decreasing quantities of serum protein. There is no evidence that age distribution of the group tested played any significant role in the ratios obtained for the I.I.

DISCUSSION

Analysis of the factors involved in the procedure showed that the coagulability of 52 per cent of the Group I sera is abnormal for entirely different reasons from the sera of Groups II and III. Evidence for this lack of homogeneity between the protein values and the degree of positive H.M.J., obtained for tubercular sera and the sera from new untreated cancer soon became apparent, i.e. the serum protein of the positive I.I. of the tubercular sera did not lie within the same range of protein per cent for the new cancer cases as for the tubercular. Furthermore,

TABLE III.—*Distribution of Iodoacetate Index for Malignant and Benign Tumours.*

Group I: Malignant tumours.	Iodacetate index.	
	<9·0.	>9·0.
Comedocarcinoma, duct glands	2	—
Sarcoma, breast	2	—
Squamous cell epithelioma lip	1	2
Adenocarcinoma nasopharynx	1	1
Lymphosarcoma	1	—
Malignant melanoma	2	—
Leukæmia, lymphatic	1	—
Adenocarcinoma ovary	1	—
,, lung, bronchogenic	1	—
,, cervix	6	6
,, breast	6	13
	24	22

Group II.	Iodoacetate index.	
	<9·0.	>9·0.
Multiple myeloma	—	2
Hodgkins	—	3
Adenocarcinoma thyroid	—	1
Lymphosarcoma, stomach	—	1
Squamous cell epithelioma mouth	1	—
Adenocarcinoma rectum	2	3
Lymphosarcoma, tonsil	1	1
Adenocarcinoma uterus	4	—
,, cervix, with metastases	6	—
,, ,,	2	1
,, breast	4	1
,, stomach	2	—
,, duodenum, with metastases	1	—
	23	13

Group IV: Benign tumours.	Iodoacetate index.	
	<9·0.	>9·0.
Basal cell carcinoma	7	2
Leukoplakia, mouth	1	4
,, vagina	—	5
Cervical polyps	—	10
,, fibrosis	2	8
Fibroid uteri	1	17
Pigmented verrucae	—	12
,, naevus	—	8
Keratosis	1	7
Sebaceous cysts	—	4
Cystic breast	2	1
	14	78

when we attempted to substitute a more rapid and less costly method for estimating the serum protein, such as a viscosimetric procedure (Kagan, 1938; Phelps and Van Slyke, 1943) our attempts met with failure when relating the I.I. to the Group I patients. In some the " positive " was altered to " negative," and in others the positive results became more or less positive. There was good agreement, however, for the I.I. values established by both Kjeldahl and falling-drop methods of protein estimations, for the remaining groups of cases. This led us to consider an " aberrant " or " combined-protein " as being responsible for the striking disagreement with the H.M.J. for the two types of disease.

The possibility that we were dealing with an increase in one of the normal fractions of the serum, the alpha globulins, reported to be increased in malignancy, led us to carry out the test on a sample of mucoprotein from cancerous serum, generously supplied by Dr. Richard Winzler, of the Department of Biochemistry, of the University of Southern California, and a " Bence Jones " protein, isolated from the urine of one of the two myeloma cases in this series. When adjusted to an equivalent of 7·0 g. per 100 ml. neither of these substances yielded a positive I.I. If, as others have observed, the degree of increase of alpha globulin is proportional to the severity of active tissue destruction, then apparently the alpha globulin plays no part in the Huggins test, either for advanced tuberculosis, which certainly involves active tissue destruction, or in the early and new untreated cancer cases in our study.

The question of the existence of modified serum protein molecules in various disease states, including malignancy, has been discussed recently by one of those whose published data established a close correlation between plasma viscosity, and the severity of the organic changes (Harkness, 1949). Added emphasis that we may be dealing with an altered protein in malignant tumours is given by the experimental observations of Hellwig (1949), who found that as a rule the size of spherical bodies in the form of clusters, short-chains and aggregates from tumour extracts when examined by electron microscope was exceedingly larger than that in benign or normal tissue. He concludes that these globules are not virus-like causative agents of cancer, but rather globular proteins, probable aggregates of the cytoplasmic globules due to an alteration in the colloidal state of the cancer cell. It is quite possible that this cytoplasmic material may be introduced into the blood serum and may play a role in accounting for the positive I.I. despite a normal range of serum protein in the 24 cases of Group I.

It is quite likely that those methods which measure the antiproteolytic activity (Clark, Cliffton and Newton, 1948), the rate of cell proliferation (Norris and Majnarich, 1948, 1949), the enzyme inhibiting or oxidation and reduction (Duboff and Hirshfeld, 1946; Savignac, Gant and Sizer, 1944) inhibition of a dye by human serum in early malignant disease involve the proteins or substances associated with the proteins of the serum.

Since the completion of this manuscript, Huggins, Jensen, Player and Hospelborn (1949) have published observations which support the theory of alteration of the serum protein molecule in malignancy, by employing the binding of a cationic dye. Only 44·7 per cent of their cases demonstrated a significantly abnormal effect.

Huggins and his colleagues have demonstrated a valuable and fundamental approach to the nature of these groups in health and disease. It is, however, well to record that their method suffers from one serious drawback—the large

subjective element in the estimation of the coagulum. The task for the future is the elimination of this factor, perhaps, by measuring the degree of coagulation by an appropriate electronic procedure characterized by the electrical resistance or potential of the coagulum.

Can one explain the paradox of the 22 negative H.M.J. results in Group I ? This question should be studied from the following approach : If the 24 positive H.M.J. tests have a pattern of deformation in one direction, it is not inconceivable that the former 22 cases have a pattern of deformation in another direction that is not manifested in the test through the present combination of reagents. Furthermore, if the mechanism of action of this method is due to the increase of net negative charges on the protein molecule by the addition of sodium iodoacetate, then our results strongly suggest variations in the polar character of the protein molecule in the serum of certain phases of tumour development.

The magnitude of relationship between absolute protein per cent and the I.I. demonstrated by Group III serum does not reflect the magnitude of the effect, when applied to Group I. The term " false positive " cannot be applied, therefore, to such tuberculosis sera, in the same sense as the term " positive " is applied to that of early cancerous sera, since the term " positive " itself is not tenable on the same basis. The serum of individuals undergoing some type of degenerative process, appears to possess an admixture of some elements, the source of which we do not yet comprehend, but the presence of which has been demonstrated by measurable function of the thermocoagulation of protein in the presence of an extrinsic inhibitor, iodoacetate.

Tentatively, we are inclined toward the conclusion that abnormality in the blood serum of some cancer patients, unlike that of some tuberculosis blood with a corresponding I.I. (but not corresponding protein concentration), appears to be the effect of wholely or partially altered configuration of protein molecules as yet unidentified.

SUMMARY.

1. Human sera from new untreated and advanced treated cancer, from advanced and arrested tuberculosis, benign growths, and non-symptomatic healthy persons were examined by a modified Iodoacetate Index Test.

2. There was a decrease in the Iodoacetate Index with corresponding decrease in the serum values for 100 per cent of the advanced tubercular and 63 per cent of the advanced cancer cases.

3. However, there was also a decrease of the Iodoacetate Index without a corresponding decrease in the serum protein for 52 per cent of the sera of new, untreated cancer, 17 per cent of the benign tumours and 1·2 per cent of the non-symptomatic controls.

4. The inhibited serum thermocoagulation phenomena involved in the Iodoacetate Index Test appears to be non-specific for active tuberculosis and advanced cancer, but appears to be specific in certain early or new untreated malignancies and benign tumour growths.

5. It is suggested that the use of the expression " false positive " or " pseudo-positive " be eliminated from the terminology of cancer tests.

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REFERENCES.

Clark, C. G. C., Cliffton, E. E., and Newton, B. L.—(1948) *Proc. Soc. exp. Biol. N.Y.*, **69**, 276.

Duboff, G. S., and Hirshfeld, S.—(1946) *Cancer Res.*, **6**, 57.

Harkness, J.—(1949) *Biochim. Biophys. Acta.*, **3**, 34.

Hellwig, C. A.—(1949) *Arch. Path.*, **48**, 426.

Huggins, C., Jensen, E. V., Player, M. A., and Hospelborn, V. D.—(1949) *Cancer Res.*, **9**, 753.

Idem, Miller, G. M., and Jensen, E. V.—(1949) *Ibid.*, **9**, 177.

Kagan, B. M.—(1938) *J. clin. Invest.*, **17**, 369.

Norris, E. R., and Majnarich, J. J.—(1948) *Amer. J. Physiol.*, **153**, 483.—(1949) *Proc. Soc. exp. Biol. N.Y.*, **70**, 229.

Phelps, R. A., and Van Slyke, D. D.—(1943) *Bull. U.S. med. Dept.*, **71**, 66.

Savignac, R. J., Gant, J. C., and Sizer, I. W.—(1944) *Amer. Ass. Adv. Sci.* Monograph on the Res. Conf. Cancer, p. 241.

THE EFFECT OF ISCHAEMIA ON THE FREE AMINO-ACID CONTENT OF TISSUES.

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VALUABLE information has accrued from comparison of the chemical characteristics of experimental neoplasms with their tissues of histogenesis (Greenstein, 1947). Many of the chemical procedures performed previously will undoubtedly be repeated on material obtained from man in order to increase our knowledge of clinical cancer. Specimens obtained from the surgical operating room seem ideal, theoretically, for a comparative study of the free amino-acids in neoplastic and normal tissues because both are obtained from the same individual under identical circumstances. It was found, however, that specimens of the same normal tissue and similar cancers from different people yielded exceedingly variable amino-acid patterns on two-dimensional paper partition chromatograms prepared in the manner first described by Consden, Gordon and Martin (1944) and elaborated by Dent (1948).

Each of seven carcinomas of the colon, for instance, contained free alanine, aspartic acid, cystine, glutamic acid, glycine, serine and valine, as did the non-cancerous colonic mucosa removed from the same surgically resected specimens. Repeated analysis of a single neoplasm or normal tissue gave highly reproducible results, but the findings among the group of carcinomas of the colon and colonic mucosas were inconsistent. Only 7 amino-acids could be identified in extracts made from one cancer, while 17 were found in extracts of another. Intermediate numbers between these two extremes were observed. There was no detectable difference between the quality of the free amino-acids isolated from a carcinoma and normal mucosa when both tissues were removed from the same surgical specimen. The same technique applied to the study of free amino-acids extractable from gastric and mammary carcinomas, and their tissues of histogenesis gave similar results. This suggested that the variability observed in these human tissues might be caused by the duration of ischaemia prior to their excision. Surgical technique frequently requires early occlusion of the blood supply to an area to be excised, and extirpation of a tumor is often accomplished only after the tissues have been ischaemic for some time.

The effect of ischaemia on the qualitative free amino-acid content of organs and tissues was investigated. Homolateral femoral, renal and spermatic arteries and the superior mesenteric artery of each of three anaesthetized rabbits which had been fasted 48 hours were occluded. Pieces of muscle, kidney, small intestine and testis were excised immediately and at intervals of 15 minutes thereafter. Similar pieces of tissue were removed at 15-minute intervals from a control rabbit

in which no vessels had been occluded to make certain that any increase in amino-acids could be attributed to ischaemia and not solely to changes in the cut surfaces of the tissues. Each specimen was frozen at once.

The frozen specimens were dried *in vacuo* and ground through a 60-mesh screen in a Wiley mill. One millilitre of 78 per cent ethanol was used for the extraction of each 100 mg. of dried, ground tissue. This proportion insured chromatograms of satisfactory color intensity. Ethanol was used for the extraction because it is believed to be a solvent which causes minimum proteolysis during denaturation of protein. A 78 per cent solution has been found to be optimal for solubility of the amino-acids and for the extraction of non-protein nitrogen only.

A two-dimensional chromatogram on Whatman No. 1 filter-paper was prepared from each of the extracts. Of 30 per cent hydrogen peroxide 100 µl. were superimposed on this spot to effect conversion of cystine and methionine to cysteic acid and methionine sulfone respectively, which yielded discrete spots on the chromatogram (Dent, 1948). Tryptophane was usually obscured by phenylalanine and the leucines. Phenol and equal parts of γ-collidine and 2,4-lutidine were the solvents used for the mobile phase.

The amino-acids that could be extracted with 78 per cent ethanol varied with the source of the tissue, but were constant over a period of one hour in those organs and tissues in which the blood supply had not been occluded. Ischaemia, however, produced a variable picture which could be correlated with the duration of anoxia. New amino-acids appeared at varying intervals in each of the four tissues studied, and the changes were consistent for each tissue in all of the rabbits studied. The results are presented in Table I. No attempt was made to determine the alpha-amino nitrogen liberated by this procedure, but the size and color intensity of the spots became greater as ischaemia was prolonged, suggesting that the concentration of those amino-acids present at the beginning of the experiment may have increased.

Although the extractable amino-acids differ with different tissues, as Roberts and Frankel (1949) have shown, the amino-acids which are liberated by acid hydrolysis from each of the tissues analysed are similar.

Proteolysis occurs under conditions of low oxygen tension and acid reaction (Maver, Johnson and Voegtlin, 1935; Engel, Harrison and Long, 1944; Russell, Long and Engel, 1944). These conditions obtain when an organ or tissue becomes ischaemic (Wallace, Dale, Laidlaw, Richards, Bayliss and Cannon, 1919; Bradley, 1922; Chen and Bradley, 1924; MacLeod, 1930). Stoner and Green (1948) reported an average increase of 123 per cent in the tyrosine content of unhydrolyzed rat muscle after the blood supply had been occluded for 5 hours. They found that muscle temperature and pH decreased with ischaemia. Proteolysis found during ischaemia may be caused by accelerated enzymic degradation in a more acid medium as the optimum pH of tissue cathepsins lies between 4 and 5 (Willstätter and Bamann, 1929). In any event ischaemia may alter the chemical characteristics of a tissue.

SUMMARY.

The number of free amino-acids extractable from clinical cancers of similar histogenesis varied widely, due apparently to deliberate occlusion of the arterial supply in the course of surgical excision. Ischaemia promoted proteolysis in

Amino-acids Extracted by 78 per cent Ethanol Related to Duration of Ischaemia in Various Tissues.

Amino-acids.	Acid hydrolysate.	Duration of ischaemia.																			
		Kidney.					Skeletal muscle.					Small intestine.					Testis.				
		0.	15.	30.	45.	60.	0.	15.	30.	45.	60.	0.	15.	30.	45.	60.	0.	15.	30.	45.	60.
Alanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine	+	0	0	0	+	+	0	0	0	0	+	+	+	+	+	+	0	0	0	0	0
Aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	0	0	0
Cystine	+	+	+	+	+	+	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+
Glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Histidine	+	+	+	+	+	+	0	0	0	+	+	0	0	0	0	+	0	+	+	+	+
Hydroxyproline	+*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leucine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lysine	+	0	0	0	+	+	0	0	0	0	+	+	+	+	+	+	0	0	+	+	+
Methionine	+	+	+	+	+	+	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+
Proline	+	+	+	+	+	+	0	0	0	0	0	+	+	+	+	+	0	0	0	0	0
Serine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Threonine	+	0	+	+	+	+	0	0	0	0	+	+	+	+	+	+	0	+	+	+	+
Tyrosine	+	0	+	+	+	+	0	0	0	0	+	0	0	+	+	+	0	0	0	0	0
Valine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

* Not found in hydrolyzed kidney.

rabbit tissues, since it increased consistently the number of free amino-acids that could be extracted from kidney, skeletal muscle, small intestine and testis.

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REFERENCES.

BRADLEY, H. C.—(1922) *Physiol. Rev.*, **2**, 415.
CHEN, K. K., AND BRADLEY, H. C.—(1924) *J. biol. Chem.*, **59**, 151.
CONSDEN, R., GORDON, A. H., AND MARTIN, A. J. P.—(1944) *Biochem. J.*, **38**, 224.
DENT, C. E.—(1948) *Ibid.*, **43**, 169.
ENGEL, F. L., HARRISON, H. C., AND LONG, C. N. H.—(1944) *J. exp. Med.*, **79**, 9.
GREENSTEIN, J. P.—(1947) ' Biochemistry of Cancer.' New York (Academic Press, Inc.).
MACLEOD, J. J. R.—(1930) ' Physiology and Biochemistry in Modern Medicine.' 6th ed. St. Louis (The C. V. Mosby Co.), p. 528.
MAYER, M. E., JOHNSON, J. M., AND VOEGTLIN, C.—(1935) *Nat. Inst. Hlth. Bull.*, **164**, 29.
ROBERTS, E., AND FRANKEL, S.—(1949) *Cancer Res.*, **9**, 645.
RUSSELL, J. A., LONG, C. N. H., AND ENGEL, F. L.—(1944) *J. exp. Med.*, **79**, 1.
STONER, H. B., AND GREEN, H. N.—(1948) *Brit. J. exp. Path.*, **29**, 121.
WALLACE, C. S., DALE, H. H., LAIDLAW, P. P., RICHARDS, A. N., BAYLISS, W. M., AND CANNON, W. B.—(1919) *Spec. Rep. Ser. med. Res. Comm.*, No. 26.
WILLSTÄTTER, R., AND BAMANN, E.—(1929) *Z. physiol. Chem.*, **180**, 127.